IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application No. 09/424,519

Applicant: Mitchell et al.

Filed: March 3, 2000

TC/AU: 1614

Examiner: Kwon, B. Y. S.

Docket No.: 175931 (DHHS Reference No. E-167-1997/0-US-07)

Customer No.: 45733

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SECOND DECLARATION UNDER 37 C.F.R. § 1.132 OF JAMES B. MITCHELL

I, James B. Mitchell, hereby declare that:

1. I am currently the Branch Chief of the Radiation Oncology Branch at the National Cancer Institute at the National Institutes of Health in Bethesda, Maryland. I have held this position since 1992. I have over 30 years of experience in the area of radiation biology, including modulation of cellular redox potential (oxidative stress), radiation sensitizers and protectors, and imaging free radicals. My Curriculum Vitae sets forth further details of my research and educational background (Exhibit A).

2. I received a Bachelor of Science Degree in Chemistry and Biology from Austin Peay State University in Clarksville, Tennessee in 1970, a Master's Degree in Biology from George Peabody College in Nashville, Tennessee in 1975, and a Ph.D. Degree in Cellular Radiation Biology from Colorado State University, Fort Collins, Colorado in 1978.

3. I am one of the named inventors in the present application. I am aware of the application and pending claims.

4. Claim 28 of the application has been rejected for obviousness-type double patenting in view of claim 22 of U.S. Patent 5,462,946 ("the '946 patent") and claim 2 of U.S. Patent 6,605,619 ("the '619 patent").

5. The Office Action argues that since radiation can cause cancer, it must follow that any agent (including Tempol) shown to protect against radiation damage should also result in the delay of onset of tumor formation. In my opinion, this contention is not true.

6. Claim 22 of the '946 patent is directed to a method for treating the effects of oxidative stress due to the production of harmful free radical species in an organism comprising administering a composition comprising Tempol. Claim 2 of the '619 patent recites a method of treating or preventing damage to normal cells, tissue, or organs in a mammal that has been exposed to ionizing radiation comprising administering to the mammal, after exposure to ionizing radiation, a composition comprising Tempol.

7. Cancer is a complex disease. It has been hypothesized that cancer results from an initiation event, followed by a series of complex promotion events (over a long period of time) that ultimately result in tumor formation (Vogelstein B, and Kinzler KW. The multistep nature of cancer. *Trends Genet* 9: 138-141, 1993; Weinberg RA. How cancer arises. *Sci Am* 275: 62-70, 1996). One might consider radiation or oxidation stress as being an "initiation event" (along with many other initiators such as toxic chemicals, air pollutants, UV light, etc.); however, the complex promotion events and mechanisms that occur over long periods of time that ultimately result in tumor formation are not known. Genetic defects, such as due to ataxia telangiectasia and Li-Fraumeni's syndrome, which are inherited conditions, can lead to tumor formation, but the complex promotion events associated with these genetic defects are not known. It is also not known whether the complex promotion events associated with radiation or oxidative stress initiation are the same as those associated with genetic defects. Because there are so many unknown variables in cancer initiation, formation, and termination mechanisms and pathways, starting from a genetic defect, the chances are minimal that one of ordinary skill in the art would have a reasonable expectation of success to arrive at the methods of the present invention. For

example, to arrive at the presently claimed invention, one of ordinary skill in the art should test an untold number of gene mutations and study pathways relating to such mutations leading to cancer. This would entail undue experimentation and hardship.

8. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

James B. Mitchell 3-22-06

James B. Mitchell, Ph.D. Date

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Exhibit A

CURRICULUM VITAE

NAME: **James B. Mitchell**

DATE AND PLACE OF BIRTH: July 2, 1948, Springfield, Tennessee

CITIZENSHIP: United States

MARITAL STATUS: Married, 3 Children

EDUCATION:

1966-1970	Austin Peay State University Clarksville, Tennessee Chemistry and Biology Bachelor of Science
1973-1975	George Peabody College Nashville, Tennessee Biology Master of Arts
1975-1978	Colorado State University Fort Collins, Colorado Cellular Radiation Biology Doctor of Philosophy

POSITIONS HELD:

November 1970-May, 1975	Research Associate Vanderbilt University Hospital Department of Radiation Therapy Nashville, Tennessee
June 1975-May, 1979	Research Associate Colorado State University Department of Radiology and Radiation Biology Fort Collins, Colorado 80521
June 1979-June, 1980	IPA Assignee Radiobiology Section Radiation Oncology Branch National Cancer Institute

	National Institutes of Health Bethesda, MD 20892
June 1980-May, 1984	Cancer Expert Radiobiology Section Radiation Oncology Branch National Cancer Institute National Institutes of Health Bethesda, MD 20892
June 1984-September, 1993	Head, Radiation Biology Section Radiation Oncology Branch National Cancer Institute National Institutes of Health Bethesda, MD 20892
September 1987-September 1993	Deputy Branch Chief Radiation Oncology Branch National Cancer Institute National Institutes of Health Bethesda, MD 20892
February 1992-March 1993	Acting Branch Chief Radiation Oncology Branch National Cancer Institute National Institutes of Health Bethesda, MD 20892
October 1993-Present	Branch Chief Radiation Biology Branch National Cancer Institute National Institutes of Health Bethesda, MD 20892
August 1997-July 1999	Acting Branch Chief Radiation Oncology Branch National Cancer Institute National Institutes of Health Bethesda, MD 20892
January 1998-Present	Senior Biomedical Research Service National Cancer Institute National Institutes of Health Bethesda, MD 20892
November 2004-Present	Acting Branch Chief Radiation Oncology Branch, NCI

MILITARY SERVICE: None

PROFESSIONAL SOCIETIES:

Radiation Research Society
American Society for Therapeutic Radiology and Oncology
American Association for Cancer Research
Society for Free Radical Biology and Medicine
Oxygen Club of Washington, D.C.

HONORS AND OTHER SPECIAL SCIENTIFIC RECOGNITION:

1995	Co-Editor of Book Lung Cancer: Principles and Practice Lippincott-Raven, 1996
1995	National Cancer Institute Equal Employment Opportunity Special Achievement Award
July 1986 - June 1990	Associate Editor - Radiation Research
1990 - Present	Associate Editor - International Journal of Radiation Oncology, Biology, Physics
1991 - Present	Editorial Board - Seminars in Radiation Oncology
1992 - 1998	Editorial Board - Radiation Oncology Investigations
1986, 1988	Program Committee - Radiation Research Society
1987	National Cancer Institute Equal Employment Opportunity Special Achievement Award
1987- 1990	Scientific Program Committee - American Society for Therapeutic Research and Oncology
1988 - 1992	NIH/NCI Radiation Study Section

HONORS AND OTHER SPECIAL SCIENTIFIC RECOGNITION, Continued:

March, 1989	Recipient of the 17th Radiation Research Award
1990 - 1993	Member, Inter Society Council for Radiation Oncology
1990 - 1995	Vice Chairman, Radiation Biology Committee, American Society for Therapeutic Radiology and Oncology
1990 - 1991	Councilor, Oxygen Club of Greater Washington, DC
1992 - 1993	President, Oxygen Club of Greater Washington, DC
1992 - 1998	Tumor Biology Committee, Radiation Therapy Oncology Group
1996-present	Associate Editor Cancer Research
1999-2000	Vice-President Elect Radiation Research Society
1997-2001	Member, DCS Promotion Tenure Review Panel National Cancer Institute
1998	National Cancer Institute Quality Work Life Award
1999	Program Committee - Radiation Research Society
1999-2001	Chair, DCS Promotion Tenure Review Panel National Cancer Institute
April, 2000	Academic Hall of Fame Honor Society of Phi Kappa Phi Austin Peay State University

HONORS AND OTHER SPECIAL SCIENTIFIC RECOGNITION, Continued:

May, 2000	Co-Editor of Book Lung Cancer: Principles and Practice, First & Second Edition

2000-2001	President-Elect Radiation Research Society
2001-2002	President Radiation Research Society
2002-2003	Past-President Radiation Research Society
February, 2001	Director's Award for Service Division of Clinical Science National Cancer Institute
June, 2001	John Yuhas Award for Outstanding Research in Radiation Biology Department of Radiation Oncology University of Pennsylvania
June, 2001 – Present	Editorial Academy International Journal of Oncology
September, 2001	Merit Award for Leadership National Cancer Institute
October, 2002 & 2004	Technology Transfer Award Center for Cancer Research, NCI
February 2005-Present	Member, NCI Animal Care and Use Committee
March 2005- Present	Member, Clinical Executive Committee, CCR, NCI
September, 2005	Federal Technology Transfer Award
October, 2005	NIH Merit Award for Outstanding Achievement in Radiation Research National Cancer Institute

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RESEARCH INTERESTS:

Effects of Fractionation and Continuous Low Dose-Rate to Mammalian Cells

Modulation of Cellular Redox Potential--Oxidative Stress

Radiation Sensitizers/Protectors

Electron Paramagnetic Resonance--Imaging Free Radicals

ADMINISTRATIVE DUTIES:

Coordinate radiation biology seminar program of visiting scientists.

Teach the ROB radiotherapy residents a full course in radiation biology.

Administrative duties of Branch Chief, Radiation Biology Branch, NCI.

Administrative duties of Acting Branch Chief, Radiation Oncology Branch, NCI.

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338. Matsumoto A, Matsumoto S, Sowers AL, Koscielniak JW, Trigg NJ, Kuppusamy P, Mitchell JB, Subramanian S, Krishna MC, Matsumoto KI. Absolute oxygen tension (pO_2) in murine fatty and muscle tissue as determined by EPR. Magn Reson Med 54: 1530-1535, 2005.

339. Sostaric JZ, Miyoshi N, Riesz P, DeGraff WG, Mitchell JB. N-alkyl glucopyranosides completely inhibit ultrasound-induced cytolysis. Free Radic Biol Med 39: 1539-1548, 2005.

340. Patel K, Chen Y, Dennehy K, Blau J, Connors S, Mendonca M, Tarpey M, Krishna MC, Mitchell JB, Welch WJ, Wilcox CS. Acute antihypertensive action of nitroxides in the spontaneously hypertensive rat. Am J Physiol Regul Integr Comp Physiol 290: R37-R43, 2006.

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GG. Transfer of the AQP1 cDNA for the correction of radiation-induced salivary hypofunction. Biochim Biophys Acta 2005.

PATENTS:

1. Mitchell JB, Samuni A, DeGraff WG, Hahn SM. "Nitroxides as protectors against oxidative stress." U.S. Patent No. 5,462,946, Issued October 31, 1995.

2. Bourg J, Mitchell JB, Mirotznik M, Roth B, Subramanian S, Krishna MC, Zablocky P. "Pulsed low-frequency EPR spectrometer and Imager." Patent No. 5,387,867 Issued February 7, 1995.

3. Bourg J, Mitchell JB, Mirotznik M, Roth B, Subramanian S, Krishna MC, Zablocky P, Pohida TJ, Smith PD, Friauf WS, Tschudin RG. "Pulsed low-frequency EPR spectrometer and Imager." Patent No. 5,502,386 Issued March 26, 1996.

4. Mitchell JB, Russo A, Krishna MC, Wink DA, Liebmann JE. "Use of nitric oxide releasing compounds as hypoxic cell radiation sensitizers." U.S. Patent No. 5,650,442, Issued July 22, 1997.

5. Wink DA, Mitchell JB, Russo A, Krishna MC, Hanbauer I, Grisham MB, Granger DN. "Use of nitric oxide releasing compounds as protective agents in ischemia reperfusion injury." U.S. Patent No. 5,789,447, Issued August 4, 1998.

6. Murugesan R, Tschudin R, Subramanian S, Mitchell JB, Krishna MC. "System and method for performing *in vivo* imaging and oxymetry by pulsed electron paramagnetic resonance." Serial No. 08/504,616. Filed 8/25/95.U.S. Patent No. Issued July, 1997.

7. Tschudin R, Murugesan R, Subramanian S, Mitchell JB, Krishna MC. "Gated radiofrequency amplifier." DHHS Reference No. E-175-96/1. Filed 8/96.Allowed: March, 1997.

8. Mitchell JB, Russo A, Krishna MC, DeGraff W, DeLuca AM, Myers L, Hahn S. Benzoates as protectors against oxidative stress and chemotherapy drug toxicity. Filed with U.S. Patent Office, March 20, 1997.

9. Mitchell JB, Russo A, Krishna MC, Wink DA, Liebmann JE. "Use of nitric oxide releasing compounds as hypoxic cell radiation sensitizers." U.S. Patent No. 5,814,667, Issued September 29, 1998.

10. Mitchell, JB, Russo, A, Krishna, MC, Wink, DA, Liebmann, JE. "Use of nitric oxide-releasing compounds to sensitize cancerous cells to chemotherapeutic agents." U.S. Patent No. 5,837,736, Issued November 17, 1998.

11. Tschudin, RG, Murugesan, R, Cherukuri, MK, Mitchell, JB, Subramanian, S. "Gated RF preamplifier for use in pulsed radiofrequency electron paramagnetic resonance and MRI." U.S. Patent No. 5,828,216, Issued October 27, 1998.

12. Mitchell, JB, Russo, A, Krishna, MC, Wink, WA, Liebmann, JE. "Use of nitric oxide releasing compounds to protect noncancerous cells from chemotherapeutic agents." U.S. Patent No. 5,840,759, Issued November 24, 1998.

13. Murugesan R, Cherukuri MK, Mitchell JB, Subramanian S, Tschudin R. "In vivo imaging and oxymetry by pulsed radiofrequency paramagnetic resonance." U.S. Patent No. 5,865,746, Issued February 2, 1999.

14. Devasahayam N, Mitchell JB, Russo A, Cook JA, Afeworki M, Tschudin R, Subramanian S, Murugesan R, Harrington F, Cherukuri MK. "Resonant structure for spatial and spectral-spatial imaging of free radical spin probes using radiofrequency time domain electron paramagnetic resonance spectroscopy." DHHS Reference No. E-166-97/0, filed May 27, 1997. Allowed February 1998.

15. Roberts AB, Ashcroft GS, Russo A, Mitchell JB. "Inhibition of SMAD3 to prevent fibrosis and improve wound healing." DHHS Reference No. 10/299,886, filed November 18, 2002, Pending.

16. Mitchell JB, Samuni A, DeGraff WG, Hahn SM. "Nitroxides as protectors against oxidative stress." Patent No. 6,605,619 Issued August 12, 2003. (CIP of Patent No. 5,462,946 Issued October 31, 1995)

17. Mitchell JB, et al. "The use of nitroxide or a prodrug thereof in the prophylactic and therapeutic treatment of cancer." DHHS Reference No. E-167-1997/0-US-07, filed March 3, 2000, US Patent Application No. 09/424,519.

INVITED PRESENTATIONS:

1. Dose-rate effects in mammalian cells. Department of Radiation Therapy, University of Arizona, Tucson, Arizona, January, 1980.

2. Effects of varying dose-rate on the mammalian cell cycle. Cytogenetics Branch, Oak Ridge National Laboratory, Oak Ridge, Tennessee, March, 1980.

3. Dose-rate effects in plateau phase mammalian cells. Department of Radiation Therapy, Southwestern Medical School, Dallas, Texas, May, 1980.

4. The radiation response of Ewing's sarcoma tumor cells and CFUc from patients receiving IV BUdR. Department of Radiation Therapy, George Washington University Hospital, Washington, D.C. May, 1982.

5. Invited Symposium Speaker: Thiols and Thermal Sensitivity. Radiation Research Society Meeting in San Antonio, Texas, March, 1983.

6. The role of glutathione and thermal sensitivity. Gray Laboratory, Northwood, England, July, 1983.

7. The role of glutathione in the cellular response to radiation and selected chemotherapy drugs. Physiology Branch, Armed Forces Radiobiological Research Institute, Bethesda, Maryland, October, 1983.

8. Clinical radiobiology developments at NIH. Mid-Atlantic Chapter of American Association of Physicists in Medicine. Bethesda, Maryland, February, 1984.

9. Invited Symposium Speaker: The *in vitro* radiobiology of human lung cancer. Workshop on Radiotherapy for Lung Cancer. The International Association for the Study of Lung Cancer. King's College, Cambridge, England, June, 1984.

10. Invited Keynote Speaker: The use of non-hypoxic cell sensitizers in radiobiology and radiotherapy. Chemical Modifiers of Cancer Treatment, Clearwater, Florida, October, 1985.

11. Invited Lecturer: The importance of cellular glutathione in the radiation and chemotherapy response. University of Rochester Cancer Center, Rochester, New York, November, 1985.

12. Invited Lecturer: Cellular and Molecular properties of human lung cancer cell lines and their relationship to radiation sensitivity. Annual meeting of the Radiological Society of North America, Chicago, Illinois, November, 1985.

13. Invited Lecturer: The role of glutathione in the radiation and chemotherapy response of human lung cancer cells. Thomas Jefferson University Hospital, Philadelphia, PA. January, 1986.

14. Invited Speaker: Modification of chemotherapy response by cellular thiol modulation. Symposium, Cellular Effects of Thiol Modulation: Radiation, Hyperthermia, and Chemotherapy, 34th Annual Radiation Research Society Meeting, Las Vegas, Nevada, April 1986.

15. Invited Speaker: The role of glutathione in radiation and drug induced cytotoxicity. 13th L.H. Gray Conference, Free Radical Biochemistry and Radiation Injury, Brunel University, London, United Kingdom, July, 1986.

16. Invited Speaker: Photodynamic Therapy of Human Ascites. Australian Phototherapy Conference, Ludwig Cancer Institute, Royal Melbourne Hospital, Melbourne, October 1986.

17. Invited Speaker: Role of Glutathione and Other Endogenous Thiols in Radiation Protection. Symposium on Perspectives in Radioprotection, Armed Forces Radiobiology Research Institute. Bethesda, Maryland, March, 1987.

18. Invited Speaker: Glutathione Levels as a Predictor of Cell Response to Drugs and Radiation. Conference on Prediction of Tumor Treatment Response. Banff, Canada .. April, 1987.

19. Invited Speaker: Comparison of Misonidazole Hypoxic Cytotoxicity Between Cell Lines with Different Intracellular Glutathione Levels and the Influence of Thiol Modulation on the Chemotherapy and Radiation Response. Eight International Congress of Radiation Research, Edinburgh Scotland, July, 1987.

20. Invited Speaker: "Biological considerations of dose rate effects." First Biennial Brachytherapy and Remote Afterloading Symposium and Workshop. Mallinckrodt Institute of Radiology. St. Louis, Missouri, September 1987.

21. Invited Speaker: "Are chemoresistant cells radioresistant?" Second International Head and Neck Oncology Research Conference. Arlington, Virginia, September 1987.

22. Refresher Course "Radiobiology and Clinical Radiation Therapy: Mechanisms of Repair". 1987 ASTRO meeting. Boston, Massachusetts. October, 1987.

23. Invited Speaker: Scientific Session: Cellular and Molecular Properties of Human Lung Cancer Cell Lines. Radiation Therapy Oncology Group, New Orleans, Louisiana, January, 1988.

24. Invited Cancer Center Speaker: Glutathione as a Predictor of Tumor Response? Yale University. New Haven, Connecticut, January, 1988.

25. Invited Keynote Speaker: Glutathione Modulation in Cancer Treatment: Will it work? Clinical Modifiers of Cancer Treatment. Paris, France. March, 1988.

26. Workshop Chairman: Hyperthermia and Oxidative Stress. Eight Annual Meeting of the North America Hyperthermia Group. Philadelphia, Pennsylvania April, 1988.

27. Invited Speaker: Bioclinical Conference, Glutathione modulation and cancer treatment. Washington University School of Medicine. St. Louis, Missouri May, 1988.

28. Invited Speaker: Department of Biochemistry Rounds: Glutathione Modulation and Cancer Treatment. University of Pennsylvania. Philadelphia, Pennsylvania, June, 1988.

29. Invited Speaker. Biological Basis of Low and High Dose Rate Brachytherapy. The VIII Brachytherapy Update 1988. Memorial Sloan-Kettering Cancer Center. New York, New York, September, 1988.

30. Invited Speaker: Refresher Course "Radiobiology and Clinical Radiation Therapy: Mechanisms of Repair". 1988 ASTRO meeting. New Orleans, Louisiana October, 1988.

31. Invited Speaker: "The Radiobiology and Clinical Application of Halogenated Pyrimidine Radiosensitizers" 15th L.H. Gray Conference, University of Kent, Canterbury, England, April, 1989.

32. Invited Speaker: "Halogenated Pyrimidine Radiosensitizers," University of Maryland Cancer Center Grand Rounds, Baltimore, Maryland, June, 1989.

33. Invited Speaker: "The Role of Glutathione in Drug and Radiation Response" International Congress of Radiology, Paris, France, July, 1989.

34. Invited Speaker: "Nitroxides as Superoxide Dismutase Mimics," Cancer Center Grand Rounds, University of Wisconsin Clinical Cancer Center, Madison, Wisconsin, July, 1989.

35. Invited Speaker: Refresher Course: "Radiobiology and Clinical Radiation Therapy: Mechanisms of Repair". 1989 ASTRO meeting. San Francisco, California, October, 1989.

36. Invited Speaker: "Glutathione Modulation and Cancer Treatment," 6th Annual Y.H. Lee Lecture, Department of Radiation Oncology, University of Minnesota, Minneapolis, Minnesota., November, 1989.

37. Invited Speaker: "Oxygen Free Radicals and Cancer Treatment." Oxygen Club of Washington, November, 1989.

38. Invited Speaker: "Nitroxide Free Radicals as Protectors against Oxidative Stress." Radiation Oncology Rounds. Henry Ford Hospital, Detroit, Michigan, May, 1991.

39. Invited Speaker: "New Radiation Protectors," Radiation Oncology Rounds, University of Pennsylvania, Philadelphia, Pennsylvania, May, 1991.

40. Invited Speaker: "The Issue of Cross Resistance Between Chemotherapy and Radiation." Educational Program of the American Society of Clinical Oncology. Twenty-seventh Annual Meeting of the American Society of Clinical Oncology, Houston, Texas, May 1991.

41. Invited Speaker: "Nitroxides as Protectors Against Oxidative Stress," New Developments in Free Radical Research - Prospects for new drugs. International Business Communications, Philadelphia, Pennsylvania, June, 1991.

42. Invited Symposium Speaker: "Prospects for Modifying the Radiosensitivity of Oxic Tumor Cells." Radiobiology of Human Tumor Cells Symposium. 9th International Congress Radiation Research, Toronto, Canada, July, 1991.

43. Invited Symposium Speaker: "Nitroxides as Protectors Against Oxidative Stress." Third International Symposium on Spin Trapping and Aminoxyl Radical Chemistry. Kyoto, Japan, November 1991.

44. Invited Symposium Speaker: "Protectors Against Oxidative Stress." 40th Annual Meeting of the Radiation Research Society, Salt Lake City, Utah, March 1992.

45. Invited Symposium Speaker: "Nitroxide Protection Against the Hypoxic Cytotoxicity of Mitomycin C and SR-4233." Bioreductives Workshop, Oxford, England, September 1992.

46. Invited Debator: "This House Believes that Current Assays for Radiosensitivity Have no Role to Play in Predicting Radiotherapy Outcome." Third Annual Painter Debate, 41st Annual Meeting of the Radiation Research Society, Dallas, Texas, March 1993.

47. Invited Speaker: "Potential Use of Nitroxides in Radiation Oncology." 4th International Conference on Anticarcinogenesis and Radiation Protection, Baltimore, Maryland, April 1993.

48. Invited Speaker: "Radiobiological considerations in radionuclide therapy." 40th Annual Meeting of the Society of Nuclear Medicine/Radiopharmaceutical Science Council, Toronto, Ontario, Canada, June 1993.

49. Invited Speaker: "Taxol-mediated G2/M cell cycle blocks and radiosensitization: differences among human tumor cell types." 8th International Conference on Chemical Modifiers, Kyoto, Japan, June 1993.

50. Invited Speaker: "Modifying the aerobic response to radiation and cytotoxic drugs." International Congress of Radiation Oncology/Kyoto '93 Meeting, Kyoto, Japan, June 1993.

51. Invited Symposium Speaker: "Recent advances in the use of radioprotectors." Semi Annual Meeting of the Radiation Therapy Oncology Group, Philadelphia, Pennsylvania, July 1993.

52. Invited Symposium Speaker: "New directions for free radical cancer research and medical applications." International Symposium: Free Radicals in Diagnostic Medicine University of New York at Buffalo, Buffalo, New York, October 1993.

53. Invited Speaker: "Fight a radical with a radical." The Joint Center for Radiation Therapy's Cancer Biology Division Seminar Series, Harvard Medical School, Boston, Massachusetts, December 1993.

54. Invited Speaker: "Radioprotection by free radicals." Radiation Protection and Radiation Recovery Research Program, University of Arkansas, Little Rock, Arkansas, February 1994.

55. Invited Speaker: "Fight a radical with a radical." Radiation Oncology Department, University of Pennsylvania, Philadelphia, PA, June 1994.

56. Invited Speaker: "Free radicals as protectors against oxidative stress." Radiation Oncology Department, Johns Hopkins Medical School, Baltimore, MD June 1994.

57. Invited Speaker: "The use of taxol as a radiation modifier." Chemoradiation Summit Meeting, Cancer Center at St. Agnes, Fresno, CA, September 1994.

58. Invited Speaker: "Free radicals as radioprotectors." ASTRO, San Francisco, CA, October 1994.

59. Invited Speaker: "Free radicals as protectors against oxidative stress." Anti-Oxidants, Oxidants, and Free Radicals, National Library of Medicine, National Institutes of Health, Bethesda, MD, December 1994.

60. Invited Speaker/Workshop Chair: "Nitric oxide and cancer treatment." Annual Meeting of the Radiation Research Society, San Jose, CA, April 1995.

61. Presentation: "Radiation sensitization by nitric oxide releasing agents." Ninth International Conference on Chemical Modifiers of Cancer Treatment, Oxford, England, August 1995.

62. Presentation: "Modulation of radiation-induced cytotoxicity by the nitric oxide releasing agent S-nitrosoglutathione (GSNO)." 10th International Congress on Radiation Research, Wurzburg, Germany, August 1995.

63. Invited Speaker: "Chemotherapy and radiation interactions." Fifth Biennial Meeting of the International Gynecological Cancer Society, Philadelphia, PA, September 1995.

64. Invited Speaker: "Chemical radiation sensitizers and protectors and normal tissue and tumor physiology." ASTRO, Miami, FL, October 1995.

65. Invited Speaker: "Nitroxides as protectors against oxidative stress." 44th Annual Meeting of the Radiation Research Society, Chicago, IL, April 1996.

66. Invited Speaker: "Free radicals as modulators of cancer treatment modalities." Grand Rounds, Duke University Medical Center, Durham, NC, November 1996.

67. Invited Speaker. "Free radicals as modulators of cancer treatment modalities." Laboratory of Biochemistry and Cell Signaling, NHLBI, NIH, January 1997.

68. Invited Symposium Speaker: "Dose, dose rate, and fractionation for vascular radiobiology." Advances In Cardiovascular Radiation Therapy, Washington, DC, February, 1997.

69. Invited Symposium Speaker: "Radioprotectant Therapies." Symposium: Radiation-Induced Xerostomia in Cancer Patients: Current Treatments and Future Therapies. International Association for Dental Research, Orlando, FL, March 1997.

70. Invited Symposium Speaker: "Nitric Oxide as a Modulator of Cancer Treatment Modalities." Symposium: Nitric Oxide: It's Role in Tumor Biology. Forty-fifth Annual Meeting of the Radiation Research Society, Providence, Rhode Island, May 1997.

71. Invited Workshop Speaker: "Imaging of Nitroxides in vivo." Workshop: Oxidative Stress and the Cellular Responses to Cancer Therapy. Forty-fifth Annual Meeting of the Radiation Research Society, Providence, Rhode Island, May 1997.

72. Invited Speaker: "Fourier Transform EPR Imaging" National Cancer Advisory Board, NIH, Bethesda, MD, June 1997.

73. Invited Speaker: "ESR Using Nitroxides to Study Free Radical Generation." Phase I Meeting Noninvasive Imaging Methods: Applications for Pharmacology, Therapeutics Development and Tumor Evaluation. NIH, Bethesda, MD September 1997.

74. Invited Speaker: "Nitroxides as Protectors Against Oxidative Stress." 10th Oxygen Radicals in Biology Gordon Research Conference, Ventura, CA, February 1998.

75. Invited Symposium Speaker: "Dose, Dose Rate and Radiation Sensitizers for Vascular Radiobiology." Advances in Cardiovascular Radiation Therapy II, Washington, DC, March 1998.

76. Invited Symposium Speaker: "Hyperthermia and Oxidative Stress Revisited," Symposium: Role of Oxidiative Stress in Biological Responses to Heat Shock. Forty-sixth Annual Meeting of the Radiation Research Society & the Seventeenth Annual Meeting of the North American Hyperthermia Society, Louisville, KY, April, 1998.

77. Invited Visiting Professor: "Oxidative Stress, Protection, and Imaging of Free Radicals," Grand Rounds, Department of Radiation Oncology, University of Maryland, School of Medicine, Baltimore, Maryland, May, 1998.

78. Invited Speaker. "Radiation, Radicals, and Images." Festschrift to Honor Dr. Daniel Gilbert, A Half Century of Radical Ideas. Sponsored by the NIH ROS Interest Group and Washington Area Oxygen Club, July 2, 1998, Bethesda, Maryland.

79. Invited Speaker: "Development of Functional Electron Paramagnetic Resonance Imaging Using Free Radical Probes." Breast Cancer Think Tank Retreat, sponsored by the National Cancer Insititue & the Division of Clinical Sciences. Westfields International Conference Center, Chantilly, Virginia, July, 1998.

80. Invited Speaker: "Principles of Chemoradiation: Theoretical and Practical Considerations." 2nd Bi-Annual Symposium on Concomitant Chemoradiotherapy, Nashville, TN, August, 1998.

81. Presentation: "Applications of EPR imaging with nitroxides to evaluate heterogeneities in tumor vs normal tissues." *In vivo* EPR and Related Studies, Dartmouth College, Hanover, NH, September 1998.

82. Invited Speaker: "Understanding Dose, Dose Rate, and Radiosensitizers." Cardiovascular Radiation Therapy for the Interventionalist. "Fundamentals of Radiation Biology for the Clinician." Radiation Vascular Therapy in Coronary and Peripheral Vascular Disease. Tenth Annual Symposium: Transcatheter Cardiovascular Therapeutics, October, 1998, Washington, D.C.

83. Invited Keynote Speaker: "Stable Nitroxide Free Radicals as Antioxidants, Radio-Protectors, and Imaging Agents." Radiation Research Laboratory, University of Iowa, 50th Anniversary Scientific Program, October, 1998, Iowa City, Iowa.

84. Invited Speaker: "Imaging of Free Radicals." ROS/RNS in Radiation-Induced Cell Injury and Disease. 5th Annual Meeting of the Oxygen Society, November, 1998, Washington, D.C.

85. Invited Speaker: "Functional Imaging Using Electron Paramagnetic Resonance." Conference of the Joint Working Group on Quantitative In Vivo Functional Imaging in Oncology, Sponsored by the U.S. Public Health Service's Office on Women's Health and the National Cancer Institute, Arlington, Virginia, January 1999.

86. Invited Speaker: "Protection Against Oxidative Stress by Nitroxides," Special Symposium, Society for Experimental Biology and Medicine and the Oxygen Club of Greater Washington, D.C., Bethesda, Maryland, February 1999.

87. Invited Symposium Speaker: "A Survey of In Vivo Antioxidant Effects of Nitroxides," Symposium: Antioxidants in Toxicology, American Chemical Society National Meeting, Anaheim, California, March, 1999.

88. Invited Speaker: "Oxidative Stress, Protection, and the Imaging of Free Radicals," Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA, April, 1999.

89. Invited Speaker: "Oxidative Stress, Protection, and the Imaging of Free Radicals," Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana, June, 1999.

90. Invited Speaker: "Stable Nitroxide Free Radicals as Antioxidants, Radiation Protectors, and Imaging Agents," In Vivo EPR Symposium, 41st Rocky Mountain Conference on Analytical Chemistry, Denver, Colorado, August, 1999.

91. Invited Speaker: "Hypoxia Modifiers, "Symposium on Organ Preservation Therapies for Squamous Cancers of the Head and Neck, National Cancer Institue, National Institute for Deafness and Other Communcation Disorders, and National Institute for Dental and Craniofacial Research, NIH, September, 1999, Bethesda, MD.

92. Invited Speaker: "Nitroxides as Protectors against Oxidative Stress," Lombardi Cancer Center's Tumor Biology Seminar Series, Georgetown University Medical Center, Washington, D.C., November, 1999.

93. Invited Speaker: "Nitroxides as Radiation Protectors,"International Conference on Low-Level Radiation Injury and Medical Countermeasures, Armed Forces Radiobiology Research Institue, Bethesda, MD, November, 1999.

94. Invited Speaker: "Nitroxide Stable Free Radicals as Radiation Protectors." Eighth Annual Radiation Workshop at Round Top, M.D. Anderson Cancer Center, Round Top, Texas, April 2000.

95. Invited Speaker: Refresher Course: Molecular Biology-"Molecular Insights into the Cancer Cell." 42nd Annual Meeting of ASTRO. Boston, Massachusetts. October, 2000.

96. Invited Speaker: Education Session: "Electron Paramagnetic Resonance and Overhauser Enhanced MRI (EPR/OMRI)- In Vivo Imaging of Oxygen," 92nd Annual Meeting of the American Association for Cancer Research. New Orleans, LA. March 2001.

97. Invited Symposium Speaker: Biological Applications: "Nitroxide Free Radicals as Protectors Against Oxidative Stress." 43rd Rocky Mountain Conference on Analytical Chemistry. Denver, Colorado. July, 2001.

98. Invited Symposium Speaker: "Nitroxides as Protectors Against Oxidative Stress." 1st Annual Photobiology Working Group, Pennsylvania College of Optometry, Philadelphia, PA. November, 2001.

99. Invited Workshop Speaker: "Nitroxide Radioprotection." Interagency Workshop: Molecular and Cellular Biology of Moderate Dose Radiation and Potential Mechanisms of Radiation Protection. Sponsored jointly by NCI, DOD, DOE, and the Radiation Research Society. December 2001, Bethesda, Maryland.

100. Invited Speaker: "Novel Magnetic Resonance Imaging for Non-Invasive Assessment of Tissue Oxygen Concentration and Redox Status." Presidential Symposium: Metabolic Oxidative Stress and Cancer Therapy, Forty-ninth Annual Meeting of the Radiation Research Society, April 2002.

101. Invited Speaker: "Novel Magnetic Resonance Imaging for Non-Invasive Assessment of Tissue Oxygen Concentration and Redox Status." Tenth Annual Radiation Workshop at Round Top, (Tumor Microenvironment: Biology and Therapeutic Implications) M.D. Anderson Cancer Center, Round Top, Texas, April 2002.

102. Invited Chair: "EPR and MR Microscopy." Tenth Annual Meeting of the International Society for Magnetic Resonance in Medicine, Hawaii Convention Center, Honolulu, Hawaii, May 2002.

103. Invited Keynote Speaker: "Nitroxides as Antioxidants." Third International Conference on Oxygen/Nitrogen Radicals: Cell Injury and Disease. Sponsored by The Centers for

Disease Control and Prevention and The National Institute for Occupational Safety and Health. Morgantown, WV, June 2002.

104. Invited Symposium Speaker: "Stable Nitroxide Free Radicals, Oxidative Stress, and Functional Imaging." 7th International Symposium on Spin Trapping 2002," The Carolina Inn, Chapel Hill, North Carolina, July 2002.

105. Invited Speaker: "New EPR Imaging: Metabolism as an Imaging Target." Second NCI Young Investigators Workshop, Bethesda, MD, August 2002.

106. Invited Faculty Speaker: "Basic Concepts of Radiation Biology: Chemistry, Fractionation, and Sensitization." Radiation Biology Refresher Course for Radiation Oncology Residents. University of Maryland, Baltimore, Maryland, August 2002.

107. Invited Consultant: "Functional Imaging and Radiation Oncology." Centre for Biological Imaging and Adaptive Radiotherapy, Cross Cancer Institute, Edmonton, Alberta, Canada, August 2002.

108. Invited Speaker: "Imaging Using Electron Paramagnetic Resonance." Oxygen Homestasis/Hypoxia Workshop, NCI Cancer Therapy Evaluation Program/Developmental Therapeutics Program, Arlington, VA, February 2003.

109. Invited Speaker: "Functional Magnetic Resonance Imaging for Determination of Tissue Oxygen and Redox Status." Vascular Biology Faculty, Center for Cancer Research, NCI, Bethesda, MD, March 2003.

110. Invited Symposium Speaker: "Novel Magnetic Resonance Imaging for Tissue Oxygen Concentration in Cancer." Sixteenth Annual meeting of the Engineering and Urology Society, Chicago, IL, April 2003.

111. Invited Speaker: "What Radiobiologists do." Fifth Grade Career Fair, Liberty Elementary School, Libertytown, MD, May 2003.

112. Invited Speaker: "Novel Functional Imaging and Radiation Protection." Prostate Cancer Support Group, National Naval Medical Center, Bethesda, MD, May 2003.

113. Invited Symposium Speaker: "Novel Functional Imaging for Tissue Oxygen Concentration and Redox Status." Free Radicals: The Pros and Cons of Antioxidants, Divisions of Prevention and Cancer Treatment and Diagnosis, National Cancer Institute, National Center for Complementary and Alternative Medicine, Bethesda, MD, June 2003.

114. Invited Speaker: "Oxidative Stress and Applications of Functional Imaging." Pediatric Oncology Branch CME Seminar Series, National Cancer Institute, Bethesda, MD, October 2003.

115. Invited Symposium Speaker: "Nitroxide Antioxidants." 3rd Annual Photobiology Working Group, Pennsylvania College of Optometry, Philadelphia, PA. November 2003.

116. Invited Speaker: "Oxidative Stress, Radioprotection, and Applications of Novel Functional Imaging Technology" and "Radiation Biology Refresher for Radiation Oncology Residents." Eastern Virginia Medical School, CME Breast Oncology Lecture Series, Norfolk, VA, March 2004.

117. Invited Symposium Speaker: "Nitroxide –Mediated Radioprotection of Normal Tissues." Radiation Response Modifiers Symposium, 51st Annual Meeting of the Radiation Research Society, St. Louis, MO, April 2004.

118. Invited Symposium Speaker: "Imaging Hypoxia in Murine Squamous Cell Carcinoma using ^{64}Cu-ATSM." Biological Imaging Symposium, 51st Annual Meeting of the Radiation Research Society, St. Louis, MO, April 2004.

119. Invited Symposium Speaker: "Magnetic Resonance Imaging Approaches for Non-Invasive Assessment of Tissue Oxygen Concentration." Presidental Symposium, 51st Annual Meeting of the Radiation Research Society, St. Louis, MO, April 2004.

120. Invited Speaker: "What Research Scientists Do." Fifth Grade Career Fair, Liberty Elementary School, Libertytown, MD, May 2004.

121. Invited Speaker: "New Class of Antioxidants (Nitroxides) for Use as Radioprotectors." NIAID Biodefense Workshop: Animal Models for Radiation Injury, Protection, and Therapy, Bethesda, MD, May 2004.

122. Invited Lecturer: "Redox Biology and Cancer Treatment." NCI Redox Biology Course. Bethesda, MD, November 23, 2004.

123. Invited Speaker: "New Class of Antioxidants (Nitroxides) for use as Radioprotectants." Radiation Protection Workshop, Defense Advanced Research Projects Agency (DARPA), Defense Sciences Office, December 13-14, 2004, Arlington, VA.

124. Invited Plenary Speaker: "Biologic Principles of Fractionation in Radiation Therapy." American College of Surgeons Oncology Group Semiannual Meeting, San Antonio, TX, January 14, 2005.

125. Invited Keynote Speaker: "Radiation Protection: Yesterday, Today, and Tomorrow." 18th Annual Meeting of the Oxygen Club of Greater Washington, Bethesda, MD, July 29, 2005.

126. Invited Plenary Lecturer: "Chasing Free Radicals: Therapeutic Applications of Nitroxides." 11th In Vivo EPR Spectroscopy and Imaging and 8th International EPR Spin Trapping Meeting, Columbus, OH, September 2005.

127. Invited Speaker: "Tissue Hypoxia Assessment using Electron Paramagnetic Resonance Imaging (EPRI)." 4th Annual NCI-RRP/ROSP Young Investigator Workshop, Bethesda, MD, September 2005.

128. Invited Speaker: "Chasing Free Radicals: Use of Nitroxide Antioxidants in Cancer Treatment and Prevention." Center for Cancer Research Grand Rounds, National Cancer Institute, Bethesda, MD, September 2005.

129. Invited Lecturer: "Chasing Free Radicals: Therapeutic Applications of Nitroxides." Department of Radiological Health Sciences, Colorado State University, Fort Collins, CO, October 2005.

130. Invited Speaker: "Novel Magnetic Resonance Approaches to Non-Invasively Measure Tissue Oxygen Concentration." NCI HIF Interest Group, Bethesda, MD, October 2005.

131. Invited Lecturer: "Redox Biology and Cancer Treatment." NCI Redox Biology Course. Bethesda, MD, November 15, 2005.

How Cancer Arises

An explosion of research is uncovering the long-hidden molecular underpinnings of cancer—and suggesting new therapies

by Robert A. Weinberg

How cancer develops is no longer a mystery. During the past two decades, investigators have made astonishing progress in identifying the deepest bases of the process—those at the molecular level. These discoveries are robust: they will survive the scrutiny of future generations of researchers, and they will form the foundation for revolutionary approaches to treatment. No one can predict exactly when therapies targeted to the molecular alterations in cancer cells will find wide use, given that the translation of new understanding into clinical practice is complicated, slow and expensive. But the effort is now under way.

In truth, the term "cancer" refers to more than 100 forms of the disease. Almost every tissue in the body can spawn malignancies; some even yield several types. What is more, each cancer has unique features. Still, the basic processes that produce these diverse tumors appear to be quite similar. For that reason, I will refer in this article to "cancer" in generic terms, drawing on one or another type to illustrate the rules that seem to apply universally.

The 30 trillion cells of the normal, healthy body live in a complex, interdependent condominium, regulating one another's proliferation. Indeed, normal cells reproduce only when instructed to do so by other cells in their vicinity. Such unceasing collaboration ensures that each tissue maintains a size and architecture appropriate to the body's needs.

Cancer cells, in stark contrast, violate this scheme; they become deaf to the usual controls on proliferation and follow their own internal agenda for reproduction. They also possess an even more insidious property—the ability to migrate from the site where they began, invading nearby tissues and forming masses at distant sites in the body. Tumors composed of such malignant cells become more and more aggressive over time, and they become lethal when they disrupt the tissues and organs needed for the survival of the organism as a whole.

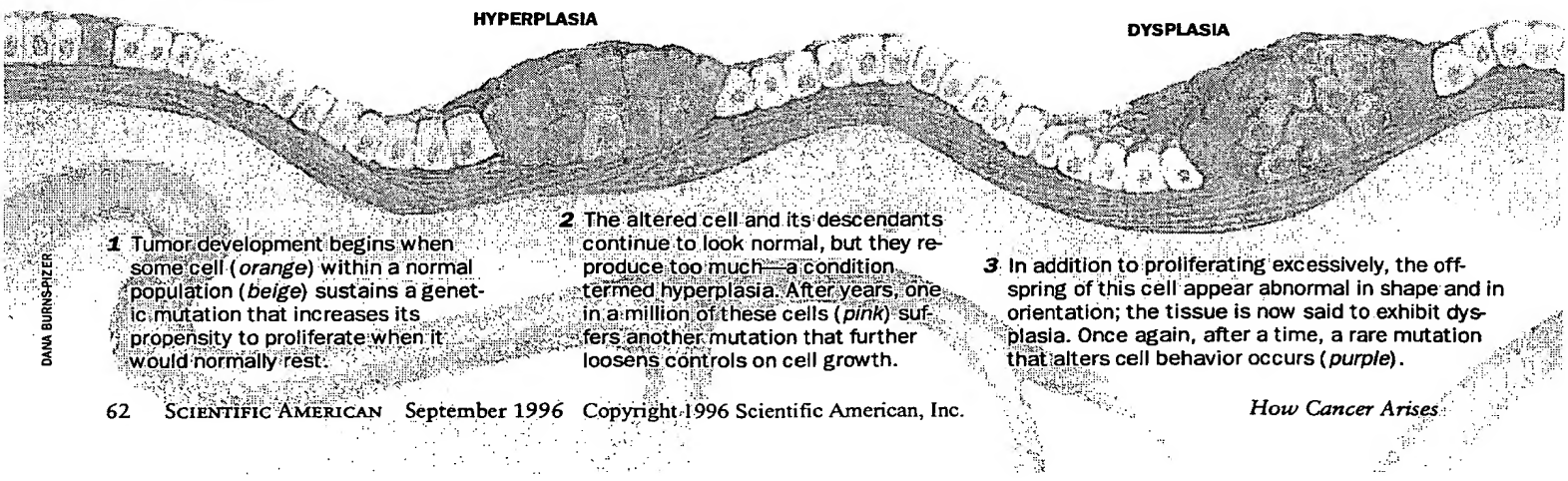
This much is not new. But over the past 20 years, scientists have uncovered a set of basic principles that govern the development of cancer. We now know that the cells in a tumor descend from a common ancestral cell that at one point—usually decades before a tumor becomes palpable—initiated a program of inappropriate reproduction. Further, the malignant transformation of a cell comes about through the accumulation of mutations in specific classes of the genes within it. These genes provide the key to understanding the processes at the root of human cancer.

Genes are carried in the DNA molecules of the chromosomes in the cell nucleus. A gene specifies a sequence of amino acids that must be linked together to make a particular protein; the protein then carries out the work of the gene. When a gene is switched on, the cell responds by synthesizing the encoded protein. Mutations in a gene can perturb a cell by changing the amounts or the activities of the protein product.

Two gene classes, which together constitute only a small proportion of the full genetic set, play major roles in triggering cancer. In their normal configuration, they choreograph the life cycle of the cell—the intricate sequence of events by which a cell enlarges and divides. Proto-oncogenes encourage such growth, whereas tumor suppressor genes inhibit it. Collectively these two gene classes ac-

Tumor Development Occurs in Stages

The creation of a malignant tumor in epithelial tissue is depicted schematically below. Epithelial cancers are the most common malignancies and are called carcinomas. The mass seen here emerges as a result of mutations in four genes, but the number of genes involved in real tumors can vary.



DANA BURNSPIZER

count for much of the uncontrolled cell proliferation seen in human cancers.

When mutated, proto-oncogenes can become carcinogenic oncogenes that drive excessive multiplication. The mutations may cause the proto-oncogene to yield too much of its encoded growth-stimulatory protein or an overly active form of it. Tumor suppressor genes, in contrast, contribute to cancer when they are inactivated by mutations. The resulting loss of functional suppressor proteins deprives the cell of crucial brakes that prevent inappropriate growth.

For a cancerous tumor to develop, mutations must occur in half a dozen or more of the founding cell's growth-controlling genes. Altered forms of yet other classes of genes may also participate in the creation of a malignancy, by specifically enabling a proliferating cell to become invasive or capable of spreading (metastasizing) throughout the body.

Signaling Systems Go Awry

Vital clues to how mutated proto-oncogenes and tumor suppressor genes contribute to cancer came from studying the roles played within the cell by the normal counterparts of these genes. After almost two decades of research, we now view the normal genetic functions with unprecedented clarity and detail.

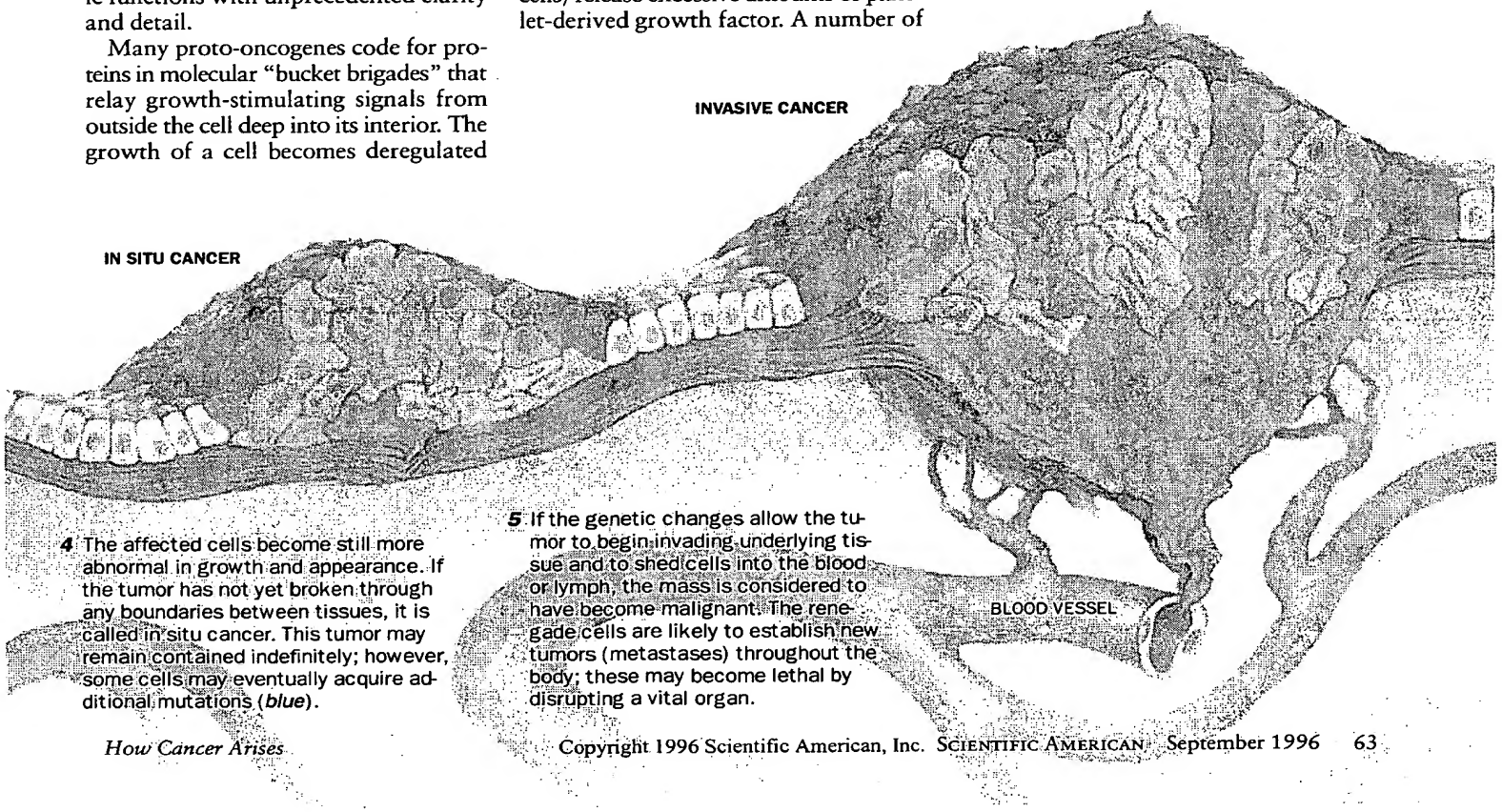
Many proto-oncogenes code for proteins in molecular "bucket brigades" that relay growth-stimulating signals from outside the cell deep into its interior. The growth of a cell becomes deregulated when a mutation in one of its proto-oncogenes energizes a critical growth-stimulatory pathway, keeping it continuously active when it should be silent.

These pathways within a cell receive and process growth-stimulatory signals transmitted by other cells in a tissue. Such cell-to-cell signaling usually begins when one cell secretes growth factors. After release, these proteins move through the spaces between cells and bind to specific receptors—antennalike molecules—on the surface of other cells nearby. Receptors span the outer membrane of the target cells, so that one end protrudes into the extracellular space, and the other end projects into the cell's interior, its cytoplasm. When a growth-stimulatory factor attaches to a receptor, the receptor conveys a proliferative signal to proteins in the cytoplasm. These downstream proteins then emit stimulatory signals to a succession of other proteins, in a chain that ends in the heart of the cell, its nucleus. Within the nucleus, proteins known as transcription factors respond by activating a cohort of genes that help to usher the cell through its growth cycle.

Some oncogenes force cells to overproduce growth factors. Sarcomas and gliomas (cancers, respectively, of connective tissues and nonneuronal brain cells) release excessive amounts of platelet-derived growth factor. A number of other cancer types secrete too much transforming growth factor alpha. These factors act, as usual, on nearby cells, but, more important, they may also turn back and drive proliferation of the same cells that just produced them.

Researchers have also identified oncogenic versions of receptor genes. The aberrant receptors specified by these oncogenes release a flood of proliferative signals into the cell cytoplasm even when no growth factors are present to urge the cell to replicate. For instance, breast cancer cells often display Erb-B2 receptor molecules that behave in this way.

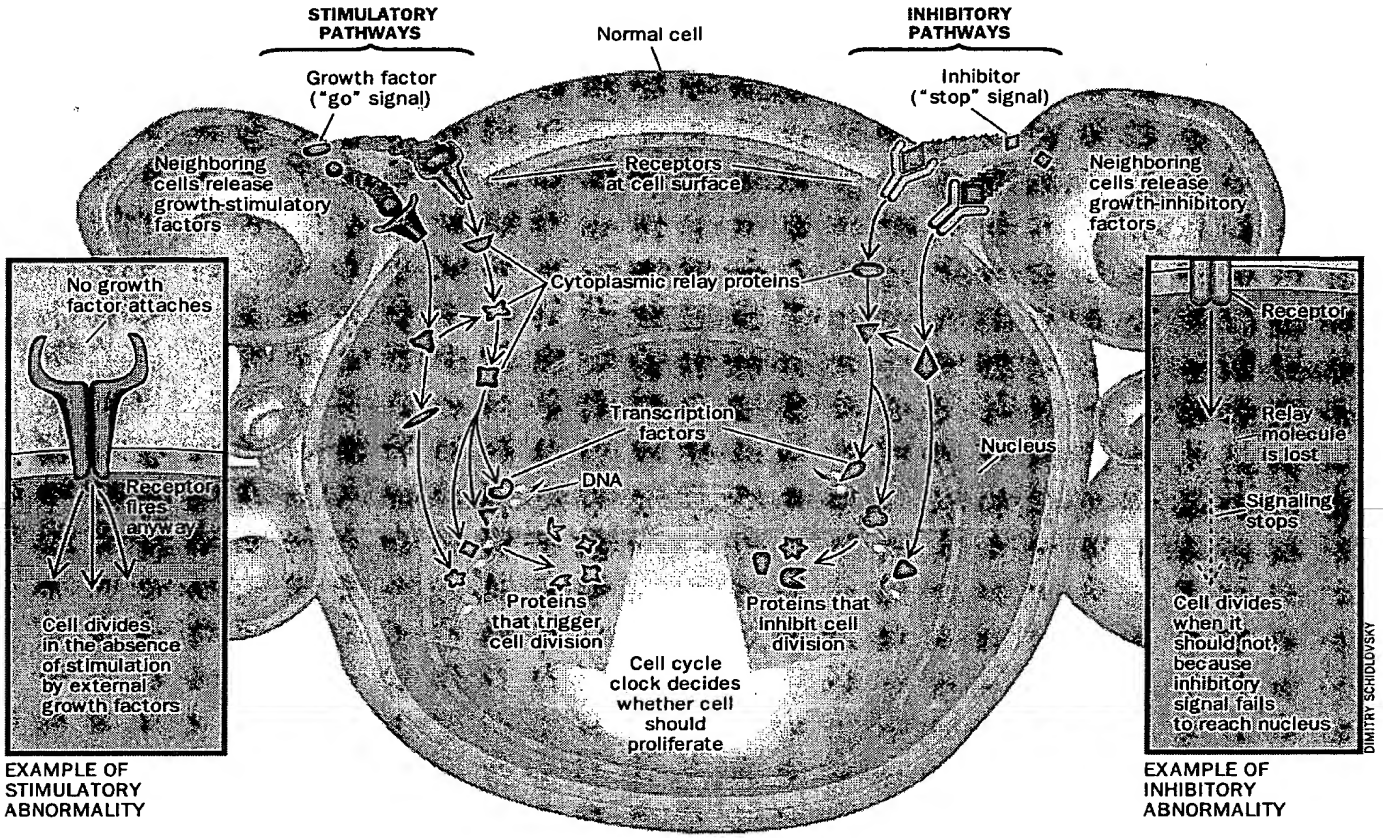
Still other oncogenes in human tumors perturb parts of the signal cascade found in the cytoplasm. The best understood example comes from the *ras* family of oncogenes. The proteins encoded by normal *ras* genes transmit stimulatory signals from growth factor receptors to other proteins farther down the line. The proteins encoded by mutant *ras* genes, however, fire continuously, even when growth factor receptors are not prompting them. Hyperactive Ras proteins are found in about a quarter of all human tumors, including carcinomas of the colon, pancreas and lung. (Carcinomas are by far the most common forms of cancer; they originate in epithelial cells, which line the body cavities



4 The affected cells become still more abnormal in growth and appearance. If the tumor has not yet broken through any boundaries between tissues, it is called in situ cancer. This tumor may remain contained indefinitely; however, some cells may eventually acquire additional mutations (*blue*).

5 If the genetic changes allow the tumor to begin invading underlying tissue and to shed cells into the blood or lymph, the mass is considered to have become malignant. The renegade cells are likely to establish new tumors (metastases) throughout the body; these may become lethal by disrupting a vital organ.

How Cancer Arises



SIGNALING PATHWAYS in normal cells convey growth-controlling messages from the outer surface deep into the nucleus. There a molecular apparatus known as the cell cycle clock collects the messages and decides whether the cell should divide. Cancer cells often proliferate excessively because genetic mutations cause stimulatory pathways (*green*) to issue too many "go" signals or because inhibitory pathways (*red*) can no longer convey "stop" signals. A stimulatory pathway will become hyperactive if a mutation causes any component, such as a growth factor receptor (*box at left*), to issue stimulatory messages autonomously, without waiting for commands from upstream. Conversely, inhibitory pathways will shut down when some constituent, such as a cytoplasmic relay (*box at right*), is eliminated and thus breaks the signaling chain.

and form the outer layer of the skin.)

Yet other oncogenes, such as those in the *myc* family, alter the activity of transcription factors in the nucleus. Cells normally manufacture Myc transcription factors only after they have been stimulated by growth factors impinging on the cell surface. Once made, Myc proteins activate genes that force cell growth forward. But in many types of cancer, especially malignancies of the blood-forming tissues, Myc levels are kept constantly high even in the absence of growth factors.

Discovery of trunk lines that carry proliferative messages from the cell surface to its nucleus has been more than intellectually satisfying. Because these pathways energize the multiplication of malignant cells, they constitute attractive targets for scientists intent on developing new types of anticancer therapeutics. In an exciting turn of events, as many as half a dozen pharmaceutical companies are working on drugs designed to shut down aberrantly firing growth factor receptors. At least three other companies are attempting to develop compounds that block the synthesis of aberrant Ras proteins. Both groups of agents halt excessive signaling in cultured cancer cells, but their utility in blocking the growth of tumors in animals and humans remains to be demonstrated.

Tumor Suppressors Stop Working

To become malignant, cells must do more than overstimulate their growth-promoting machinery. They must also devise ways to evade or ignore braking signals issued by their normal neighbors in the tissue. Inhibitory messages received by a normal cell flow to the nucleus much as stimulatory signals do—via molecular bucket brigades. In cancer cells, these inhibitory brigades may be disrupted, thereby enabling the cell to ignore normally potent inhibitory signals at the surface. Critical components of these brigades, which are specified by tumor suppressor genes, are absent or inactive in many types of cancer cells.

A secreted substance called transforming growth factor beta (TGF-ß) can stop the growth of various kinds of normal cells. Some colon cancer cells become oblivious to TGF-ß by inactivating a gene that encodes a surface receptor for this substance. Some pancreatic cancers inactivate the *DPC4* gene, whose protein product may operate downstream of the growth factor receptor. And a variety of cancers discard the *p15* gene, which codes for a protein that, in re-

sponse to signals from TGF-ß, normally shuts down the machinery that guides the cell through its growth cycle.

Tumor suppressor proteins can also restrain cell proliferation in other ways. Some, for example, block the flow of signals through growth-stimulatory circuits. One such suppressor is the product of the *NF-1* gene. This cytoplasmic molecule ambushes the Ras protein before it can emit its growth-promoting directives. Cells lacking *NF-1,* then, are missing an important counterbalance to Ras and to unchecked proliferation.

Various studies have shown that the introduction of a tumor suppressor gene into cancer cells that lack it can restore a degree of normalcy to the cells. This response suggests a tantalizing way of combating cancer—by providing cancer cells with intact versions of tumor suppressor genes they lost during tumor development. Although the concept is attractive, this strategy is held back by the technical difficulties still encumbering gene therapy for many diseases. Current procedures fail to deliver genes to a large proportion of the cells in a tumor. Until this logistical obstacle is surmounted, the use of gene therapy to cure cancer will remain a highly appealing but unfulfilled idea.

The Clock Is Struck

Over the past five years, impressive evidence has uncovered the destination of stimulatory and inhibitory pathways in the cell. They converge on a molecular apparatus in the cell nucleus that is often referred to as the cell cycle clock. The clock is the executive decision maker of the cell, and it apparently runs amok in virtually all types of human cancer. In the normal cell, the clock integrates the mixture of growth-regulating signals received by the cell and decides whether the cell should pass through its life cycle. If the answer is positive, the clock leads the process.

The cell cycle is composed of four stages. In the G_1 (gap 1) phase, the cell increases in size and prepares to copy its DNA. This copying occurs in the next stage, termed S (for synthesis), and enables the cell to duplicate precisely its complement of chromosomes. After the chromosomes are replicated, a second gap period, termed G_2, follows during which the cell prepares itself for M (mitosis)—the time when the enlarged par-

Some Genes Involved in Human Cancers

Genes known as proto-oncogenes code for proteins that stimulate cell division; mutated forms, called oncogenes, can cause the stimulatory proteins to be overactive, with the result that cells proliferate excessively. Tumor suppressor genes code for proteins that inhibit cell division. Mutations can cause the proteins to be inactivated and may thus deprive cells of needed restraints on proliferation. Investigators are still trying to decipher the specific functions of many tumor suppressor genes.

ONCOGENES

Genes for growth factors or their receptors	
PDGF	Codes for platelet-derived growth factor. Involved in glioma (a brain cancer)
erb-B	Codes for the receptor for epidermal growth factor. Involved in glioblastoma (a brain cancer) and breast cancer
erb-B2	Also called *HER-2* or *neu.* Codes for a growth factor receptor. Involved in breast, salivary gland and ovarian cancers
RET	Codes for a growth factor receptor. Involved in thyroid cancer
Genes for cytoplasmic relays in stimulatory signaling pathways	
Ki-ras	Involved in lung, ovarian, colon and pancreatic cancers
N-ras	Involved in leukemias
Genes for transcription factors that activate growth-promoting genes	
c-myc	Involved in leukemias and breast, stomach and lung cancers
N-myc	Involved in neuroblastoma (a nerve cell cancer) and glioblastoma
L-myc	Involved in lung cancer
Genes for other kinds of molecules	
Bcl-2	Codes for a protein that normally blocks cell suicide. Involved in follicular *B* cell lymphoma
Bcl-1	Also called *PRAD1.* Codes for cyclin D1, a stimulatory component of the cell cycle clock. Involved in breast, head and neck cancers
MDM2	Codes for an antagonist of the p53 tumor suppressor protein. Involved in sarcomas (connective tissue cancers) and other cancers

TUMOR SUPPRESSOR GENES

Genes for proteins in the cytoplasm	
APC	Involved in colon and stomach cancers
DPC4	Codes for a relay molecule in a signaling pathway that inhibits cell division. Involved in pancreatic cancer
NF-1	Codes for a protein that inhibits a stimulatory (Ras) protein. Involved in neurofibroma and pheochromocytoma (cancers of the peripheral nervous system) and myeloid leukemia
NF-2	Involved in meningioma and ependymoma (brain cancers) and schwannoma (affecting the wrapping around peripheral nerves)
Genes for proteins in the nucleus	
MTS1	Codes for the p16 protein, a braking component of the cell cycle clock. Involved in a wide range of cancers
RB	Codes for the pRB protein, a master brake of the cell cycle. Involved in retinoblastoma and bone, bladder, small cell lung and breast cancer
p53	Codes for the p53 protein, which can halt cell division and induce abnormal cells to kill themselves. Involved in a wide range of cancers
WT1	Involved in Wilms' tumor of the kidney
Genes for proteins whose cellular location is not yet clear	
BRCA1	Involved in breast and ovarian cancers
BRCA2	Involved in breast cancer
VHL	Involved in renal cell cancer

ent cell finally divides in half to produce its two daughters, each of which is endowed with a complete set of chromosomes. The new daughter cells immediately enter G_1 and may go through the full cycle again. Alternatively, they may stop cycling temporarily or permanently.

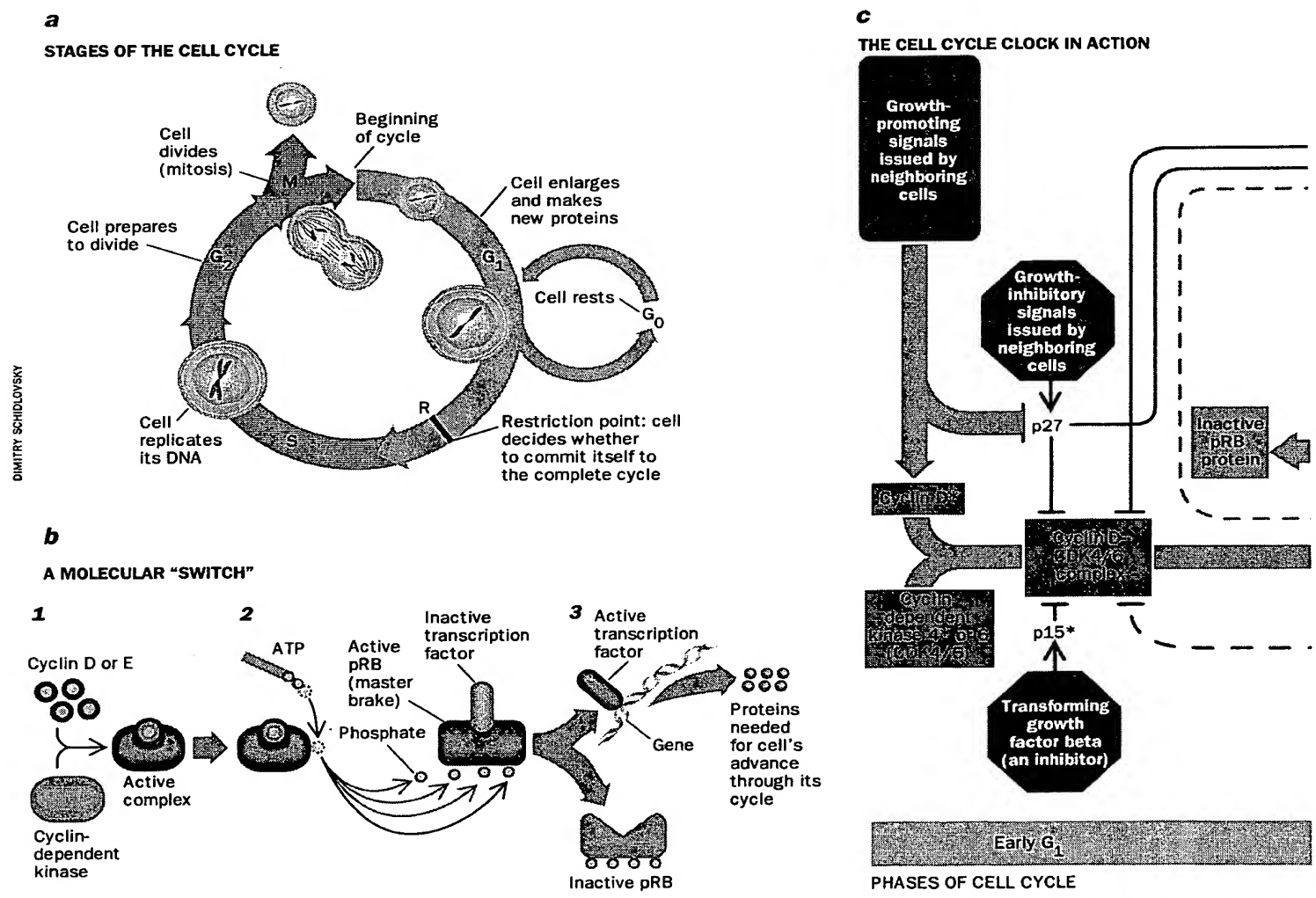
The cell cycle clock programs this elaborate succession of events by means of a variety of molecules. Its two essential components, cyclins and cyclin-dependent kinases (CDKs), associate with one another and initiate entrance into the various stages of the cell cycle. In G_1, for instance, D-type cyclins bind to CDKs 4 or 6, and the resulting complexes act on a powerful growth-inhibitory molecule—the protein known as pRB. This action releases the braking effect of pRB and enables the cell to progress into late G_1 and thence into S (DNA synthesis) phase [*see b in box below*].

Various inhibitory proteins can restrain forward movement through the cycle. Among them are p15 (mentioned earlier) and p16, both of which block the activity of the CDK partners of cy-

The Cell Cycle Clock and Cancer

Most, perhaps all, human cancers grow inappropriately not only because signaling pathways in cells are perturbed but also because the so-called cell cycle clock becomes deranged. The clock—composed of an assembly of interacting proteins in the nucleus—normally integrates messages from the stimulatory and inhibitory pathways and, if the stimulatory messages win out, programs a cell's advance through its cycle of growth and division. Progression through the four stages of the cell cycle (*a*) is driven to a large extent by rising levels of proteins called cyclins: first the D type, followed by E, A and then B.

A crucial step in the cycle occurs late in G_1 at the restriction point (R), when the cell decides whether to commit itself to completing the cycle. For the cell to pass through R and enter S, a molecular "switch" must be flipped from "off" to "on." The switch works as follows (*b*): As levels of cyclin D and, later, cyclin E rise, these proteins combine with and activate enzymes called cyclin-dependent kinases (*1*). The kinases (acting as part of cyclin-kinase complexes) grab phosphate groups (*2*) from molecules of

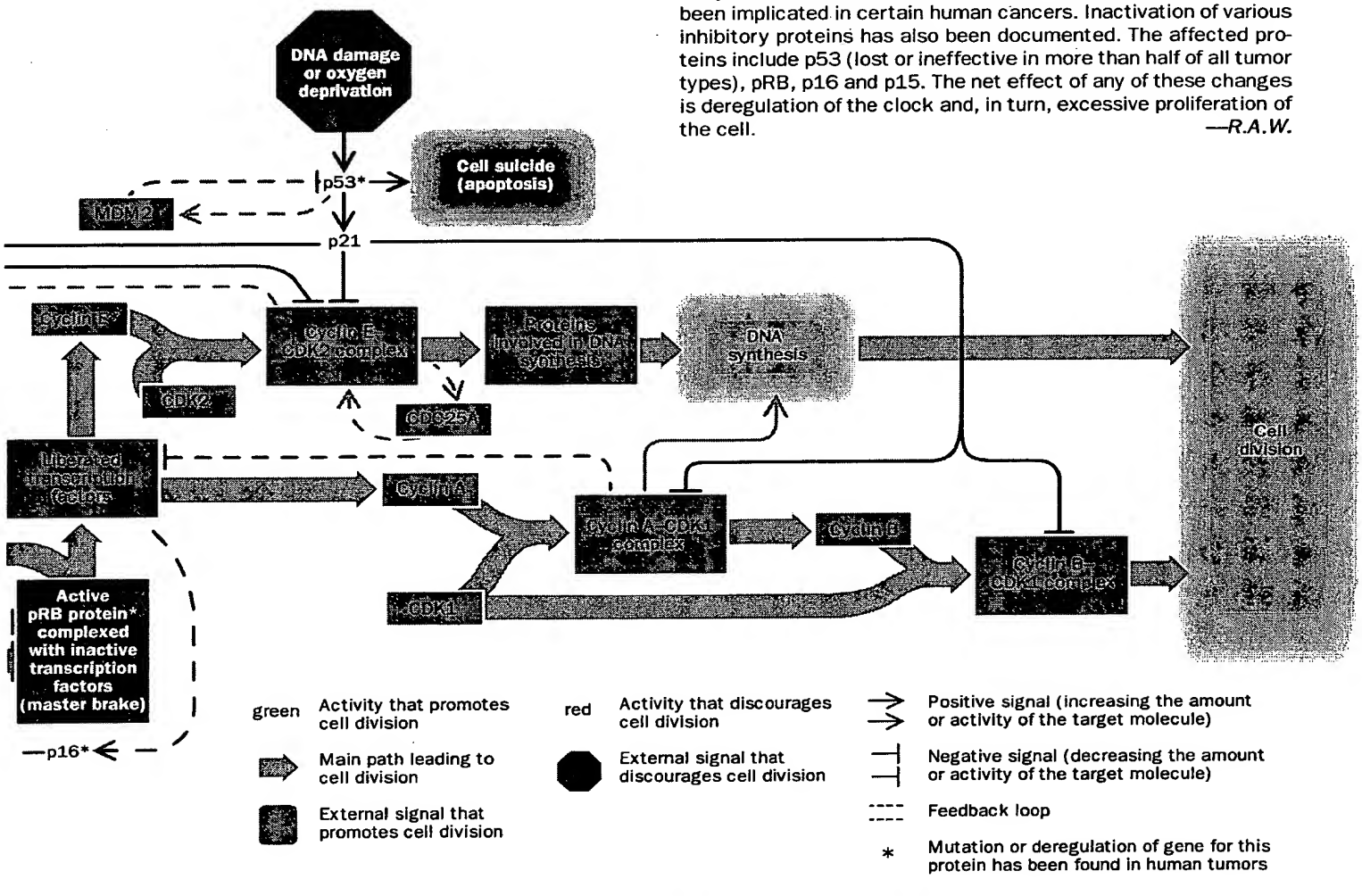


clin D, thus preventing the advance of the cell from G_1 into S. Another inhibitor of CDKs, termed p21, can act throughout the cell cycle. P21 is under control of a tumor suppressor protein, p53, that monitors the health of the cell, the integrity of its chromosomal DNA and the successful completion of the different steps in the cycle.

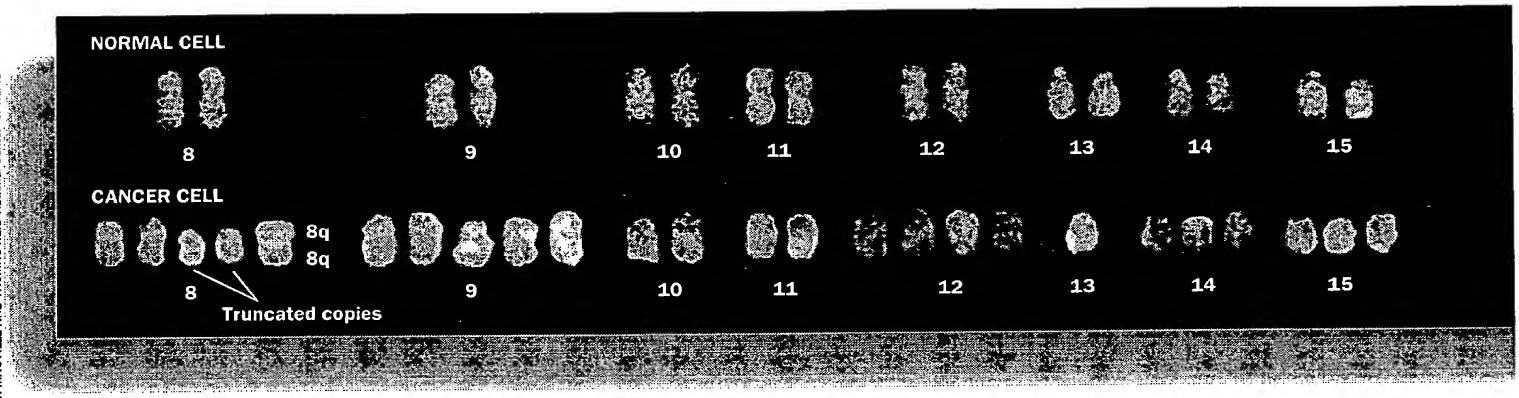
Breast cancer cells often produce excesses of cyclin D and cyclin E. In many cases of melanoma, skin cells have lost the gene encoding the braking protein p16. Half of all types of human tumors lack a functional p53 protein. And in cervical cancers triggered by infection of cells with a human papillomavirus, both the pRB and p53 proteins are frequently disabled, eliminating two of the clock's most vital restraints. The end result in all these cases is that the clock begins to spin out of control, ignoring any external warnings to stop. If investigators can devise ways to impose clamps on the cyclins and CDKs active in the cell cycle, they may be able to halt cancer cells in their tracks.

ATP (adenosine triphosphate) and transfer them to a protein called pRB, the master brake of the cell cycle clock. When pRB lacks phosphates, it actively blocks cycling (and keeps the switch in the "off" position) by sequestering other proteins termed transcription factors. But after the cyclin-kinase complexes add enough phosphates to pRB, the brake stops working (*3; bottom*); it releases the factors, freeing them to act on genes (*3; top*). The liberated factors then spur production of various proteins required for continued progression through the cell cycle.

In figure *c* below, the switch is placed in the larger context of the many molecular interactions that regulate the cell cycle. Flipping of the switch to "on" can be seen above the R point. Overactivity of the stimulatory proteins cyclin D, cyclin E and CDK4 have been implicated in certain human cancers. Inactivation of various inhibitory proteins has also been documented. The affected proteins include p53 (lost or ineffective in more than half of all tumor types), pRB, p16 and p15. The net effect of any of these changes is deregulation of the clock and, in turn, excessive proliferation of the cell. *—R.A.W.*



I have so far discussed two ways that our tissues normally hold down cell proliferation and avoid cancer. They prevent excess multiplication by depriving a cell of growth-stimulatory factors or, conversely, by showering it with antiproliferative factors. Still, as we have seen, cells on their way to becoming cancerous often circumvent these controls: they stimulate themselves and turn a deaf ear to inhibitory signals. Prepared for such eventualities, the human body equips cells with certain backup systems that guard against runaway division. But additional mutations in the cell's genetic repertoire can overcome even these defenses and contribute to cancer.



MERRYN MACVILLE AND THOMAS REID National Center for Human Genome Research, NIH

HUMAN CHROMOSOMES from a normal dividing cell (*top*) occur as identical pairs; those numbered 8 to 18 are shown. Chromosomes from a cervical cancer cell, in contrast, display many abnormalities (*bottom*). Chromosome 8, for instance, exhibits three disturbances: gain of copy number; deletion of genetic material from individual copies; and breakage followed by joining of segments that do not belong together (*far right in 8*). Copy loss, as in chromosome 13, is also common. These various changes can favor tumor progression if they activate an oncogene, increase the copies of an oncogene or eliminate a tumor suppressor gene. The images were generated by spectral karyotyping, a new method for analyzing chromosomes.

Fail-Safe Systems Fail

One such backup system, present in each human cell, provokes the cell to commit suicide (undergo "apoptosis") if some of its essential components are damaged or if its control systems are deregulated. For example, injury to chromosomal DNA can trigger apoptosis. Further, recent work from a number of laboratories indicates that creation of an oncogene or the disabling of a tumor suppressor gene within a cell can also induce this response. Destruction of a damaged cell is bad for the cell itself but makes sense for the body as a whole: the potential dangers posed to the organism by carcinogenic mutations are far greater than the small price paid in the loss of a single cell. The tumors that emerge in our tissues, then, would seem to arise from the rare, genetically disturbed cell that somehow succeeds in evading the apoptotic program hardwired into its control circuitry.

Developing cancer cells devise several means of evading apoptosis. The p53 protein, among its many functions, helps to trigger cell suicide; its inactivation by many tumor cells reduces the likelihood that genetically troubled cells will be eliminated. Cancer cells may also make excessive amounts of the protein Bcl-2, which wards off apoptosis efficiently.

Recently scientists have realized that this ability to escape apoptosis may endanger patients not only by contributing to the expansion of a tumor but also by making the resulting tumors resistant to therapy. For years, it was assumed that radiation therapy and many chemotherapeutic drugs killed malignant cells directly, by wreaking widespread havoc in their DNA. We now know that the treatments often harm DNA to a relatively minor extent. Nevertheless, the affected cells perceive that the inflicted damage cannot be repaired easily, and they actively kill themselves. This discovery implies that cancer cells able to evade apoptosis will be far less responsive to treatment. By the same token, it suggests that therapies able to restore a cell's capacity for suicide could combat cancer by improving the effectiveness of existing radiation and chemotherapeutic treatment strategies.

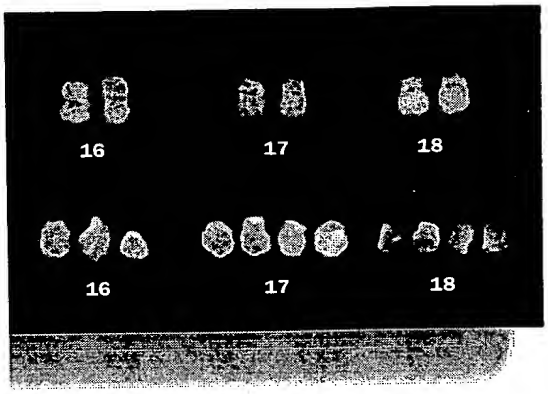
A second defense against runaway proliferation, quite distinct from the apoptotic program, is built into our cells as well. This mechanism counts and limits the total number of times cells can reproduce themselves.

Cells Become Immortal

Much of what is known about this safeguard has been learned from studies of cells cultured in a petri dish. When cells are taken from a mouse or human embryo and grown in culture, the population doubles every day or so. But after a predictable number of doublings—50 to 60 in human cells—growth stops, at which point the cells are said to be senescent. That, at least, is what happens when cells have intact *RB* and *p53* genes. Cells that sustain inactivating mutations in either of these genes continue to divide after their normal counterparts enter senescence. Eventually, though, the survivors reach a second stage, termed crisis, in which they die in large numbers. An occasional cell in this dying population, however, will escape crisis and become immortal: it and its descendants will multiply indefinitely.

These events imply the existence of a mechanism that counts the number of doublings through which a cell population has passed. During the past several years, scientists have discovered the molecular device that does this counting. DNA segments at the ends of chromo-

somes, known as telomeres, tally the number of replicative generations through which cell populations pass and, at appropriate times, initiate senescence and crisis. In so doing, they circumscribe the ability of cell populations to expand indefinitely [see "Telomeres, Telomerase and Cancer," by Carol W. Greider and Elizabeth H. Blackburn; SCIENTIFIC AMERICAN, February].



Like the plastic tips on shoelaces, the telomere caps protect chromosomal ends from damage. In most human cells, telomeres shorten a bit every time chromosomes are replicated during the S phase of the cell cycle. Once the telomeres shrink below some threshold length, they sound an alarm that instructs cells to enter senescence. If cells bypass senescence, further shrinkage of the telomere will eventually trigger crisis: extreme shortening of the telomeres will cause the chromosomes in a cell to fuse with one another or to break apart, creating genetic chaos that is fatal to the cell.

If the telomere-based counting system operated properly in cancerous cells, their excessive proliferation would be aborted long before tumors became very large. Dangerous expansion would be stemmed by the senescence program or, if the cell evaded that blockade, by disruption of the chromosomal array at crisis. But this last defense is breached during the development of most cancer cells, overcome by activation of a gene that codes for the enzyme telomerase.

This enzyme, virtually absent from most healthy cell types but present in almost all tumor cells, systematically replaces telomeric segments that are usually trimmed away during each cell cycle. In so doing, it maintains the integrity of the telomeres and thereby enables cells to replicate endlessly. The resulting cell immortality can be troublesome in a couple of ways. Obviously, it allows tumors to grow large. It also gives precancerous or already cancerous cells time to accumulate additional mutations that will increase their ability to replicate, invade and ultimately metastasize.

From the point of view of a cancer cell, production of a single enzyme is a clever way to topple the mortality barrier. Yet dependence on one enzyme may represent an Achilles' heel as well. If telomerase could be blocked in cancer cells, their telomeres would once again shrink whenever they divided, pushing these cells into crisis and death. For that reason, a number of pharmaceutical firms are attempting to develop drugs that target telomerase.

Why Some Cancers Appear Early

It normally takes decades for an incipient tumor to collect all the mutations required for its malignant growth. In some individuals, however, the time for tumor development is clearly compressed; they contract certain types of cancer decades before the typical age of onset of these cancers. How can tumor formation be accelerated?

In many cases, this early onset is explained by the inheritance from one or the other parent of a mutant cancer-causing gene. As a fertilized egg begins to divide and replicate, the set of genes provided by the sperm and egg is copied and distributed to all the body's cells. Now a typically rare event—a mutation in a critical growth-controlling gene—becomes ubiquitous, because the mutation is implanted in all the body's cells, not merely in some randomly stricken cell. In other words, the process of tumor formation leapfrogs over one of its early, slowly occurring steps, accelerating the process as a whole. As a consequence, tumor development, which usually requires three or four decades to reach completion, may culminate in one or two. Because such mutant genes can pass from generation to generation, many members of a family may be at risk for the early development of cancer.

An inherited form of colon cancer provides a dramatic example. Most cases of colon cancer occur sporadically, the results of random genetic events occurring during a person's lifetime. In certain families, however, many individuals are afflicted with early-onset colonic tumors, preordained by an inherited gene. In the sporadic cases, a rare mutation silences a tumor suppressor gene called *APC* in an intestinal epithelial cell. The resulting proliferation of the mutant cell yields a benign polyp that may eventually progress to a malignant carcinoma. But defective forms of *APC* may pass from parents to children in certain families. Members of these families develop hundreds, even thousands of colonic polyps during the first decades of life, some of which are likely to become transformed into carcinomas.

The list of familial cancer syndromes that are now traceable directly to inheritance of mutant tumor suppressor genes is growing. For instance, inherited defective versions of the gene for *pRB* often lead to development of an eye cancer—retinoblastoma—in children; later in life the mutations account for a greatly increased risk of osteosarcomas (bone cancers). Mutant inherited versions of the *p53* tumor suppressor gene yield tumors at multiple sites, a condition known as the Li-Fraumeni syndrome (named in part for Frederick Li, co-author of "What Causes Cancer?", page 80). And the recently isolated *BRCA1* and *BRCA2* genes seem to account for the bulk of familial breast cancers, encompassing as many as 20 percent of all premenopausal breast cancers in this country and a substantial proportion of familial ovarian cancers as well.

Early onset of tumors is sometimes explained by inheritance of mutations in another class of genes as well. As I implied earlier, most people avoid cancer until late in life or indefinitely because they enter the world with pristine genes. During the course of a lifetime, however, our genes are attacked by carcinogens imported into our bodies from the environment and also by chemicals produced in our own cells. And genetic errors may be introduced when the enzymes that replicate DNA during cell cycling make copying mistakes. For the most part, such errors are rapidly corrected by a repair system that operates in every cell. Should the repair system slip up and fail to erase an error, the damage will become a permanent mutation in one of the cell's genes and in that same gene in all descendant cells.

The system's high repair efficiency is one reason many decades can pass before all the mutations needed for a ma-

lignancy to develop will, by chance, come together within a single cell. Certain inherited defects, though, can accelerate tumor development through a particularly insidious means: they impair the operation of proteins that repair damaged DNA. As a result, mutations that would normally accumulate slowly will appear with alarming frequency throughout the DNA of cells. Among the affected genes are inevitably those controlling cell proliferation.

Such is the case in another inherited colon cancer, hereditary nonpolyposis colon cancer. Afflicted individuals make defective versions of a protein responsible for repairing the copying mistakes made by the DNA replication apparatus. Because of this impairment, colonic cells cannot fix DNA damage efficiently; they therefore collect mutations rapidly, accelerating cancer development by two decades or more. People affected by another familial cancer syndrome, xeroderma pigmentosum, have inherited a defective copy of a gene that directs the repair of DNA damaged by ultraviolet rays. These patients are prone to several types of sunlight-induced skin cancer.

Similarly, cells of people born with a defective *ATM* gene have difficulty recognizing the presence of certain lesions in the DNA and mobilizing the appropriate repair response. These people are susceptible to neurological degeneration, blood vessel malformation and a variety of tumors. Some researchers have proposed that as many as 10 percent of inherited breast cancers may arise in patients with a defective copy of this gene.

Over the next decade, the list of cancer susceptibility genes will grow dramatically, one of the fruits of the Human Genome Project (which seeks to identify every gene in the human cell). Together with the increasingly powerful tools of DNA analysis, knowledge of these genes will enable us to predict which members of cancer-prone families are at high risk and which have, through good fortune, inherited intact copies of these genes.

Beyond Proliferation

Although we have learned an enormous amount about the genetic basis of runaway cell proliferation, we still know rather little about the mutant genes that contribute to later stages of tumor development, specifically those that allow tumor cells to attract blood vessels for nourishment, to invade nearby tissues and to metastasize. But research in these areas is moving rapidly. (Judah Folkman describes the ingenuity of tumor cells in generating their own blood supply in "Fighting Cancer by Attacking Its Blood Supply," on page 150. Erkki Ruoslahti takes up metastasis in "How Cancer Spreads" on page 72.)

We are within striking distance of writing the detailed life histories of many human tumors from start to life-threatening finish. These biographies will be written in the language of genes and molecules. Within a decade, we will know with extraordinary precision the succession of events that constitute the complex evolution of normal cells into highly malignant, invasive derivatives.

By then, we may come to understand why certain localized masses never progress beyond their benign, noninvasive form to confront us with aggressive malignancy. Such benign growths can be found in almost every organ of the body. Perhaps we will also discern why certain mutant genes contribute to the formation of some types of cancer but not others. For example, mutant versions of the *RB* tumor suppressor gene appear often in retinoblastoma, bladder carcinoma and small cell lung carcinoma but are seen only occasionally in breast and colon carcinomas. Very likely, many of the solutions to these mysteries will flow from research in developmental biology (embryology). After all, the genes that govern embryonic development are, much later, the sources of our malignancies.

By any measure, the amount of information gathered over the past two decades about the origins of cancer is without parallel in the history of biomedical research. Some of this knowledge has already been put to good use, to build molecular tools for detecting and determining the aggressiveness of certain types of cancer, as David Sidransky discusses in "Advances in Cancer Detection," on page 104. Still, despite so much insight into cause, new curative therapies have so far remained elusive. One reason is that tumor cells differ only minimally from healthy ones; a minute fraction of the tens of thousands of genes in a cell suffers damage during malignant transformation. Thus, normal friend and malignant foe are woven of very similar cloth, and any fire directed against the enemy may do as much damage to normal tissue as to the intended target.

Yet the course of the battle is changing. The differences between normal and cancer cells may be subtle, but they are real. And the unique characteristics of tumors provide excellent targets for intervention by newly developed drugs [see the section "Therapies of the Future," beginning on page 135]. The development of targeted anticancer therapeutics is still in its infancy. This enterprise will soon move from hit-or-miss, serendipitous discovery to rational design and accurate targeting. I suspect that the first decade of the new century will reward us with cancer therapies that earlier generations could not have dreamed possible. Then this nation's long investment in basic cancer research will begin to pay off handsomely. SA

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The multistep nature of cancer

BERT VOGELSTEIN AND KENNETH W. KINZLER

Cancer is a distinct type of genetic disease in which not one, but several, mutations are required. Each mutation drives a wave of cellular multiplication associated with gradual increases in tumor size, disorganization and malignancy. Three to six such mutations appear to be required to complete this process.

One of the most important developments in genetics over the past decade has been the proof that cancer is, in essence, a genetic disease. However, there are two key differences between cancer and most other genetic diseases. First, cancer is, for the most part, caused by somatic mutations, whereas all other genetic diseases of mammals (excluding those involving mitochondrial genes) are caused solely by germ-line mutations. Second, each individual cancer arises not from a single mutation, but from the accumulation of several mutations. This 'multi-hit' concept is central to understanding neoplasia, but only in the past decade has it become possible to provide support for this concept at the molecular level. Here, we review some of the many studies that have substantiated this view.

Epidemiology

If one plots the incidence of most common human cancers against age, a striking relationship is observed: the incidence rate increases dramatically (10^3–10^7 times) with age (Fig. 1). Although there are many possible explanations for the exponential relationship, the most attractive is that three to seven 'hits' are required for a cancer to form[1]. These 'hits' could represent insults to separate cells, but because each cancer appears to arise from a single cellular progenitor (clonal growth) it is more likely that they represent sequential mutations of growth-regulatory genes in a single cell and its progeny.

According to this idea, tumors grow by a process of clonal evolution driven by mutation[2]. The first mutation would result in limited expansion of the progeny of a single cell. One of these cells would later acquire a second mutation, perhaps allowing growth of a small benign tumor. One cell within this benign tumor would then undergo a third mutation, overgrow its sister cells, and form a more advanced tumor composed of progeny cells with three mutations. Eventually the cell will accumulate a sufficient number of hits to make it malignant, enabling it to invade surrounding tissues and metastasize to other organs (the latter properties alone distinguish malignant tumors, cancers, from benign tumors). In this sequential multi-hit model of carcinogenesis, the fact that most cancers occur in older people is explained by the decades required for an individual to accumulate the number of mutations necessary to cause malignancy.

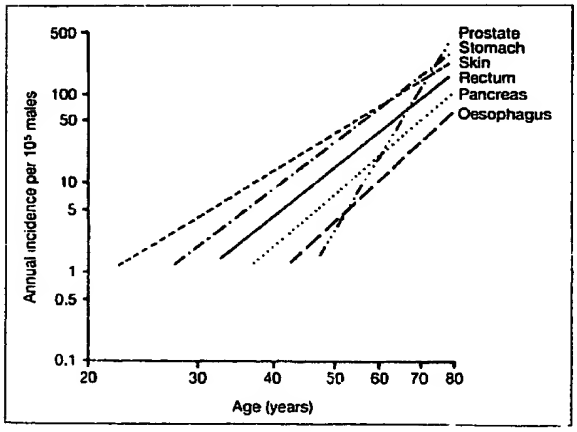

FIG 1

Cancer incidence versus age. The log of the incidence rate and the log of age have a linear relationship, with the incidence increasing according to a power of age. Modified from Ref. 1.

Other epidemiological and clinical observations were critical in formulating the multi-hit hypothesis. For example, patients exposed to radiation often develop cancer, but the cancers do not form immediately. In the case of patients who underwent X-ray therapy for tuberculosis, breast cancers develop an average of 15 years after the initial exposure[3]. Why the long time lag? One explanation is that the radiation induced a mutation in a cell, but additional mutations in the progeny of this cell were required for a cancer to form. Again, because of the low incidence of additional mutations after radiation therapy had ended, long periods were required for the cancer to appear.

Morphological observations are also in accord with the multi-hit hypothesis. In the colon, the gradual evolution of tumors is well documented. Small benign tumors (adenomas) are the first manifestations of neoplasia in colorectal epithelium. These tumors are only a few millimeters in diameter and are almost normal in their intra- and intercellular organization. With time, these tumors grow and their cells become more disorganized. Eventually, the tumor evolves into a cancer (carcinoma), presumably because one of the cells in the adenoma has acquired a sufficient number of mutations to drive the processes of invasion and metastasis. These morphological observations on colonic tumors have now been supplemented by documentation of the mutations that are associated with initiation and progression[4]. For example, it has been shown that malignant cells within a single tumor have the same set of mutations found in benign portions of the tumor, but with the addition of at least one further mutation that is absent in the benign precursor cells. A similar evolution of morphology has been observed in cervical carcinomas. The cervical smear test is used to detect neoplastic cells shed from the cervix at a stage before full-blown malignancy. Surgery at this stage can be life-saving, whereas once the tumor has acquired the ability to metastasize through sequential mutation, the resultant cancers are often incurable.

The examples given above suggest that multiple mutations occurring over decades drive the neoplastic process. Exceptions to this general scenario provide

important lessons. Tumors that occur in children obviously do not take decades to develop. The timing of tumorigenesis in such cases stimulated Knudson to propose his now well-accepted model for neoplastic development[5]. The model invokes two important principles. First, in childhood tumors of the eye and kidney, only two mutations are rate limiting for cancer formation. Second, either the two mutations can both develop somatically, or one can be inherited and the other somatic. In the latter case, every eye or kidney cell of the individual has a 'head start' on the neoplastic process, and such individuals have a high risk of developing these specific cancers. One important finding from these pediatric studies is that the number of hits required is likely to vary in different cell types. Similarly, it may vary in different species. For example, rodent cells are generally easier to transform than human cells, and this may be because fewer hits are required.

Transformation *in vitro*

The morphological and epidemiological observations noted above only indirectly support the multihit scenario. More direct evidence is provided by gene transfer experiments. An oncogene can be operationally defined as a gene whose activity leads to enhanced cell growth. If an oncogene is transferred to cultured primary rodent fibroblasts under standard conditions, no changes in growth are observed. However, if two oncogenes are transferred simultaneously, the recipient cells grow abnormally well *in vitro*, forming foci of piled-up cells. These foci are tumorigenic when implanted into mice[6]. Moreover, not all combinations of oncogenes will transform cells, so that oncogenes can be classified by their ability to complement one another in transformation assays. This suggests that the cell has evolved several growth control circuits, and more than one circuit must be damaged before abnormal growth ensues. Thus, an oncogene that disrupts one circuit can be complemented in transformation assays by a gene that disrupts a second circuit, but if both oncogenes act through the same circuit the cell maintains sufficient control to prevent neoplastic transformation.

An interesting new example of this synergy involves c-*myc* and *bcl-2*. Overexpression of c-*myc* is often associated with neoplastic growth. However, c-*myc* overexpression also has another effect: while cells grown under limiting conditions, such as serum starvation, are normally blocked at G0 or G1 in the cell cycle, cells that overexpress c-*myc* undergo apoptosis under these conditions. Overexpression of *bcl-2* can rescue these cells from premature death[7,8]. Tumor cells *in vivo* must frequently be in situations where growth factors are at low concentration, for example, in poorly vascularized areas or near necrotic portions of tumors. This may explain why alterations at the *bcl-2* locus often accompany c-*myc* overexpression in lymphomas. It is likely that other combinations of oncogenes act in a similar way to alter the complex balance between cell division and cell death that determines whether a subpopulation of tumor cells will expand.

One exception to the requirement for two separate oncogenes to transform primary cells is informative.

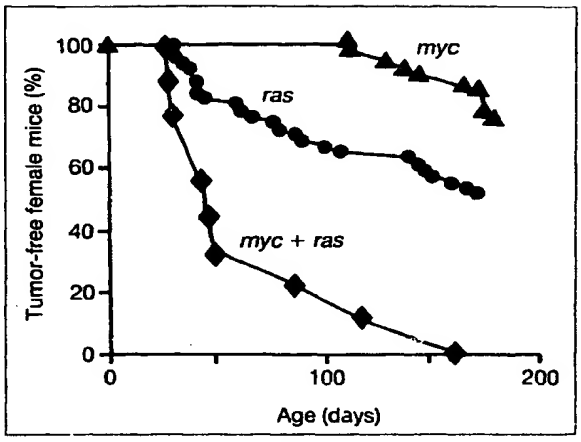

FIG 2

Oncogene synergism *in vivo*. Kinetics of breast tumor occurrence in mice transgenic for the *ras* or *myc* oncogene, and in mice doubly transgenic for *ras* and *myc*. Expression of *ras* and *myc* was under the control of the mouse mammary tumor virus breast-specific promoter. Values for t_{50}, the age by which 50% of mice had developed tumors, are as follows: *myc* transgenics, 325d (n = 50); *ras* transgenics, 168d (n = 52); *myc* + *ras* transgenics, 46d (n = 9). Reproduced, with permission, from Ref. 13.

When oncogenes are transfected together with genes encoding drug resistance, only cells that have incorporated the resistance markers can form colonies in the presence of drugs. Under these conditions, a single oncogene is sufficient for neoplastic transformation of the selected colonies[9]. It seems that in normal cells, cell–cell contact inhibits the growth of cells expressing a single oncogene, but if this cell contact inhibition is removed (by killing normal cells with a drug) one constraint on abnormal growth is removed and full transformation ensues[10]. Thus, the cellular environment (as well as the cell type and species) can modulate the number of hits required for tumorigenicity. One would expect, from these observations, that mutations that disrupt cell interactions might play an important role in promoting the growth of some cancers. This expectation has been realized: some mutations seen in human cancers reduce the expression of cell adhesion molecules, and artificial modulation of cell adhesion can promote tumor cell growth and/or invasion[11].

Tumors in transgenic animals

Studies using transgenic mice have made an important contribution to our understanding of cancer over the past decade. When an oncogene such as *myc* is transferred to the mouse germ line under the control of a breast-cell specific promoter, the transgenic animals develop breast tumors[12]. However, of the thousands of breast tumor stem cells in the mouse, only one or two become neoplastic. This suggests that the presence of a single oncogene is not sufficient for tumorigenesis, even when the gene is expressed at constitutively high levels for long periods. However, doubly transgenic mice, made by breeding *myc* transgenics with mice

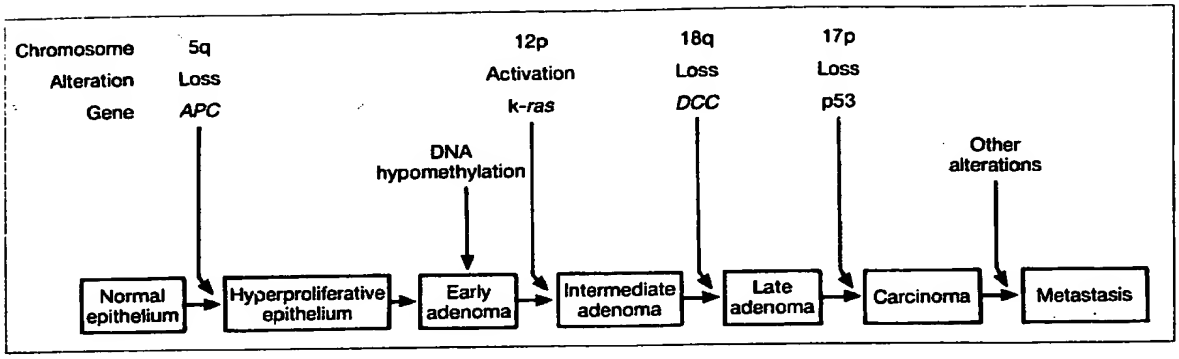

FIG 3

A genetic model for colorectal tumorigenesis. Modified from Ref. 4.

transgenic for a second breast-specific oncogene, develop tumors much earlier and more frequently (Fig. 2). Again, two oncogenes are more efficient than one. Even in the doubly transgenic mice, however, the proportion of breast cells that become neoplastic is still small, suggesting that further mutations are required to convert a normal breast epithelial cell into a cancerous one[13]. It will be of interest to determine how many transgenic oncogenes are required to convert all breast epithelial cells to malignancy, independent of additional somatic mutation.

Tumors in humans

Studies of human tumors have added a new dimension to this story. In animals, the tumorigenic process has been analysed primarily in tumors deliberately induced by mutagenic agents or viruses. In humans, dissection of the process has proceeded through the characterization of mutations occurring in sporadic tumors, often involving genes identified by positional cloning approaches. Part of the revolution in cancer research in the 1980s was the discovery that the two approaches converge: the same kinds of genes are mutated in all tumors.

Much of the research on human cancers has centered on tumor suppressor genes. These genes are negative regulators of cellular proliferation and their inactivation by mutation results in the loss of a crucial 'brake' on tumor growth. To date, six presumptive suppressor genes, each mutated in a different spectrum of human cancers, have been identified: the retinoblastoma gene, *RB* (chromosome 13q); the Wilms' tumor gene, *WT1* (11p); the gene deleted in colon carcinoma, *DCC* (18q); the neurofibromatosis type 1 gene, *NF1* (17q); the p53 gene (17p); and the gene involved in familial adenomatous polyposis coli, *APC* (5q). Several other suppressor genes have been localized to specific chromosomal regions by linkage analysis in kindreds with inherited tumor predisposition syndromes or by the pattern of somatic chromosomal losses in tumors[14]. Such genes can be mutationally inactivated in the germ line, resulting in a predisposition to tumors, or, more commonly, undergo somatic mutation, leading to initiation or progression of sporadic tumors.

A good illustration of how the studies of experimental systems and human tumors have converged, and an example of particular relevance to the multi-hit phenomenon, is provided by human papilloma viruses (HPVs). These viruses have at least two oncogenes (*E6* and *E7*) that can transform appropriate recipient cells in culture. Infection of human cervical cells with these viruses *in vivo* appears to be the first step in the pathway towards cervical cancer[15]. Infection may actually represent a double hit, because the E6 and E7 proteins bind to, and presumably inactivate, the products of two suppressor genes (the p53 and RB proteins, respectively). Only those HPV subtypes whose E6 and E7 proteins bind RB and p53 are associated with cancers in humans[16,17]. In cervical tumors that are not associated with HPV infection, p53 (and perhaps RB) may be inactivated by mutations, such as deletions, splice-site changes and codon substitutions, rather than by binding to a viral protein[18]. Progression of HPV-initiated cells to the fully malignant state requires additional hits in genes not yet identified.

Another example of the interaction between experimental and human tumor research is the characterization of chronic myelogenous leukemia (CML). One of the viruses that causes leukemia in mice carries a transduced and mutated version of a cellular gene, c-*abl*. The mutation results in constitutive activity of the abl tyrosine kinase. In CML in humans, the *ABL* gene on chromosome 9 is translocated to a locus on chromosome 22 (Ref. 19). This translocation results in the activation of the ABL tyrosine kinase, and is an early (but perhaps not the first[20]) genetic event in the development of CML. Additional genetic alterations, largely undefined at the molecular level, are required to convert the relatively indolent CML to the more aggressive forms that kill the patient; this is yet another example of the need for multiple hits in cancer evolution.

Numerous other common human tumors, including those of the breast, brain, lung, bladder, bone and colon, have been shown to have mutations at more than one gene. Because colon tumors evolve through well-defined morphological stages, it has been possible to establish the order in which mutations occur in this tumor type (Fig. 3). The development of colorectal tumors appears to be initiated by mutations at the *APC* tumor suppressor gene. These mutations usually cause truncation of the protein. It is not yet clear whether the second hit is a mutation at the remaining *APC* allele,

resulting in the total absence of functional gene product, or a mutation of some other, as yet unidentified, gene. Mutation of *APC* can occur somatically, causing initiation of a single colorectal tumor[21], or in the germ line, resulting in a predisposition to such tumors[22,23]. Patients who have a germ-line mutation of *APC* develop thousands of tumors throughout their colon; this is a remarkable demonstration of what can happen when a brake controlling cell growth is lost. Mutation at *APC* leads to the formation of benign adenomas which gradually grow bigger. Mutation of the *RAS* gene often occurs in one of these benign tumor cells, leading to a further clonal expansion. Sequential mutations in the *DCC* and p53 tumor suppressor genes appear to complete the process, driving waves of clonal expansion that finally result in progression from the benign to the malignant state. No stage of tumorigenesis is static, including the malignant (carcinomatous) stage; additional mutations occur, giving rise to small subpopulations that may not overgrow the entire tumor. These clonal subsets are one of the most difficult challenges in clinical oncology, as they are a reservoir of genetically heterogeneous cells with varying capacities for growth, differentiation and metastasis, and differential sensitivities to drugs, radiation and immune attack.

In all of the human tumors discussed above, mutations in one particular gene appear to precede those in others. Because the oncogenes and tumor suppressor genes so far identified appear to control different cell growth circuits, one might expect that the order in which the circuits are interrupted would be unimportant, and that so long as a sufficient number of critical pathways were disabled, tumor growth would ensue. In fact, although there is some variability in the order of mutations, there is also a clear preference. In CML, colorectal tumors and bladder neoplasias, respectively, mutations at *ABL* (Ref. 19), *APC* (Ref. 21), and a gene on chromosome 9q (Ref. 24) clearly precede other mutations. This suggests that only a subset of genetic pathways can initiate the tumorigenic process in particular cell types and that mutation at some genes confers a selective growth advantage only at later stages in tumor development. Moreover, in most epithelial cells, the neoplastic pathways appear to be guarded by suppressor genes, rather than oncogenes. The reverse may be true in hematopoietic cells, where mutation of an oncogene often appears to be the initiating event in neoplasia. Mutations at *RAS*, although powerful in some situations, appear to have little effect on the neoplastic transformation of epithelial cells in the absence of other critical changes. This explains why mutations of *RAS* in human tumors always occur as a late event that promotes tumor progression, rather than an early event that initiates the process.

Prospects

One of the beauties of genetics as a science is the clear cause and effect relationships that can be inferred, relating genotype to phenotype. The genetics of cancer forces us to re-examine our simple notions of causality, such as those embodied in Koch's postulates; how does one come to grips with words like 'necessary' and 'sufficient' when more than one mutation is required to produce a phenotype and when that phenotype can be produced by different mutant genes in various combinations? Similar problems arise in the analysis of other complex human diseases, such as those involving autoimmunity, atherosclerosis, hypertension and psychiatric disorders. Perhaps the conceptual lessons derived from the study of cancer will also apply to these other conditions.

On the positive side, it should be emphasized that an appreciation of the complexity and multiplicity of genetic events is the first step towards understanding these common and often lethal diseases. Moreover, multiple mutations provide multiple targets for intervention. Indeed, it has been shown that when a single normal gene or chromosomal region is introduced into a cancer cell with multiple mutations, cell growth and/or invasion can be dramatically inhibited, at least in the test tube[25]. While it is likely to be some time before we can successfully apply this type of therapy to human cancers *in vivo*, it may eventually be possible to develop drugs that will mimic the effect of normal suppressor genes or interfere with the effect of mutant oncogenes. A highlight of the last decade was the discovery of many of the genes that are responsible for human cancer. The next decade should see the characterization of the biochemical and physiological mechanisms that underlie the action of these genes, facilitating novel approaches to both therapy and prevention.

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